ON THE FUNCTION OF HAEMOGLOBIN IN *CHIRONOMUS*

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(With Three Text-figures)

I. INTRODUCTION

IN the past the ability of many chironomid larvae to survive in conditions of low oxygen concentration has attracted attention, and this ability has generally been considered to be related to their possession of haemoglobin. This view has, however, been based on insufficient experimental evidence. Miall & Hammond (1900) and Pause (1919) were of the opinion that the larvae migrated up to the surface every night, and that in the well-aerated surface waters the haemoglobin became oxygenated and carried a store sufficient to last the animal for the following day. Leitch (1916) has shown that this view is incorrect, for the haemoglobin can only carry enough oxygen to last the animal about 12 min. at 20° C. Nevertheless, Zavrel (1920) is of the opinion that the haemoglobin acts as an oxygen store to be utilized by the animal in periods of shortage.

Leitch (1916) has also shown that *in vivo* the blood becomes reduced at an oxygen pressure of 2·9 mm. of mercury at 17° C.; below this point, therefore, the haemoglobin cannot function in oxygen transport, as it will be permanently in the reduced condition. She claims also to have shown that there is no reduction of the haemoglobin above 7 mm. of mercury, but her method is not sufficiently accurate to demonstrate this, and her lower limit (2·9 mm.) only is reliable.

The function of haemoglobin in *Chironomus thummi* larvae has been studied experimentally by Harnisch (1936), making use of the carbon monoxide method. The respiration of untreated animals is compared with that of animals in which oxygen carriage by the haemoglobin has been prevented by treatment with carbon monoxide. Any differences in respiratory rate found should therefore give a measure of the amount of oxygen carried by the pigment. Harnisch has studied both normal animals ('O_2 animals') and animals which have previously been subjected to severe oxygen lack ('N_2 animals'). He finds that in the former case at air saturation the haemoglobin is not used in oxygen transport, but comes into action only at low oxygen concentrations. In the case of N_2 animals the rate of oxygen consumption is higher than that of normal animals, and he concludes from his experiments that the haemoglobin is functional in oxygen transport at all oxygen concentrations from air saturation downwards. This work of Harnisch is, however, open to numerous criticisms. In one series of experiments the temperature varied from 18·5 to 22° C.

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He appears to have discarded many experiments on the grounds that they di not give the result he expected. For instance, he states that at one time it wa impossible to obtain material which could be regarded as O_2 animals even afte prolonged aeration: 'dies verbot sowohl die Grössenordnung des Sauerstoff verbrauchs als auch sein Verhalten gegenüber Veränderung des Partialdrucks: i beidem zeigte solches Material das Verhalten von N_2-Tieren, so dass die an ihr vorgenommenen Messungen verworfen werden mussten' (1936, p. 393).

Harnisch also failed to take into account the possible direct effect of carbo monoxide on cellular oxidations. In all his experiments 20% carbon monoxide wa used, but since the amount of oxygen varied from 80 to 0·8% the relative pressur of carbon monoxide increased from 0·25 to 25 as the oxygen concentration decreased It was found that the difference between the respiratory rate of carbon monoxide treated animals and untreated animals increased as the oxygen concentration de creased; but this may have been due not to the elimination of the haemoglobin, bu to a direct effect of the carbon monoxide, becoming more marked as the relativ pressure of carbon monoxide was increased. Another objection to the work is tha two different series of readings on untreated N_2 animals give, at air pressure, value for the respiratory rate which differ from each other very nearly as much as do th values for CO animals and for untreated animals. No explanation of the differen values found for different sets of untreated animals is given.

The work of Harnisch cannot therefore be accepted as proving that the haemo globin is normally active in oxygen transport only at oxygen pressures below ai saturation, and it was thought necessary to carry out a further investigation of th function of chironomid haemoglobin. This work has had to be abandoned owing t the war, and any complete study of the subject must be extended to cover muc more of the ecology of the animals and their behaviour in circumstances as near t natural conditions as is possible. A comparison of the respiratory physiology an ecology of species with and without haemoglobin is also very desirable.

II. MATERIAL AND METHODS

The material used consisted of haemoglobin-containing chironomid larvae of th *C. plumosus* group which were obtained from Mr L. Haig, of Beam Brook, Newdi gate, Surrey. Animals were kept before an experiment in aerated water in shallo dishes of water with a thin layer of mud. In these conditions they pupated an emerged successfully.

During the course of the experiments the general appearance of the larva suggested to me that the material contained two closely related species. Adults wer therefore bred out and sent to the late Dr F. W. Edwards, F.R.S., of the Britis Museum, who kindly identified them as two species of the *C. plumosus* grou probably *C. cingulatus* Mg. and *C. riparius* Mg. As specific limits within the grou are very hard to determine it is not possible to be more definite.

The method used resembles that of Harnisch; that is to say, the respiration o normal animals is compared with that of animals treated with carbon monoxide a a series of oxygen concentrations from air saturation down to a low value.

The respiration of the chironomids was measured in water in a chamber which consisted of a 10 c.c. all glass hypodermic syringe (Fig. 1). During an experiment the animals, usually four, are placed in water in the syringe, and the nozzle stopped with a piece of glass rod. The whole is immersed and slowly rotated in a thermostatic water-bath at the desired temperature. At intervals a sample of water is withdrawn from the syringe and its oxygen content determined by the syringe pipette micro-Winkler method described by Fox & Wingfield (1938). Since the respiratory chamber is in the form of a syringe its plunger will slide in to compensate for the water sample withdrawn, and so a series of readings can be made without disturbing the animals or bringing air into contact with the water in the experimental vessel. Samples for analysis were generally withdrawn at hourly intervals and a single experiment as a rule lasted for four hours. The diminution in volume after the withdrawal of each sample is allowed for in the calculation.

When using the Winkler method of oxygen estimation in respiratory studies it is necessary to make sure that the animals are not liberating reducing substances such as nitrite or ferrous iron (Allee & Oesting, 1934). In the present case ferrous

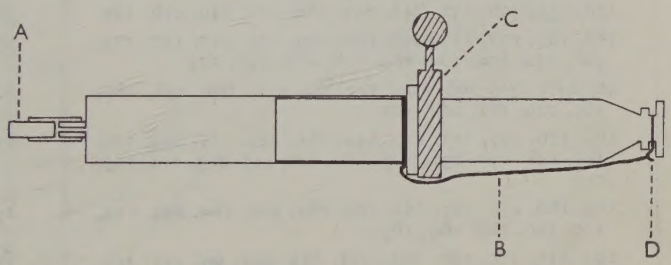

Fig. 1. Respiratory vessel. *A*, glass rod closing syringe nozzle. *B*, rubber band attached to wire hook *D*. *C*, spring clip fitted with rubber tubing to syringe plunger. The band and clip prevent any movement of the plunger between readings, and are removed when withdrawing a sample for analysis.

iron was shown to be absent, but slight traces of nitrite were found to be present occasionally in the water at the end of an experiment. The amount of nitrite present was therefore estimated colorimetrically as described in Standard Methods of Water Analysis of the American Public Health Association (1936). It was found that in no case was a concentration of more than 0·005 mg. of nitrite nitrogen per litre present in the water at the end of an experiment. This does not exceed the value which Allee & Oesting estimate as undesirable, and the use of the unmodified Winkler method was therefore regarded as legitimate.

The method of treatment with carbon monoxide was as follows. The animals were kept in water containing 1·5 c.c. of carbon monoxide and 7 c.c. of oxygen per litre (relative pressure of carbon monoxide *ca.* 1/5) for 3 hr. in darkness. The experiments were then carried out in water containing a very small amount of carbon monoxide, namely 0·2 c.c. per litre, which was sufficient to prevent dissociation of the CO compound, for it was found that if the animals were placed in water containing no carbon monoxide the haemoglobin did not always consist entirely of the CO compound at the end of an experiment.

All experiments were carried out at 17° C. in the dark.

At the end of an experiment the animals were removed from the syringe, dried on filter-paper and weighed. In the CO experiments the blood of the animals was then tested spectroscopically by the method described by Ewer & Fox (1940) to make sure that the haemoglobin had been converted to the CO compound.

III. RESULTS

The results of the first series of experiments are given in Tables 1 and 2 and Fig. 2. The values found for the respiratory rate, expressed as cu. mm. of oxygen per g. wet weight per hour, have been grouped into classes differing

Table 1. *Oxygen consumption of* Chironomus *at* 17° C. *at various concentrations of dissolved oxygen. First series: Untreated animals*

Oxygen concentration c.c./l.	Oxygen consumption cu. mm./g. (wet)/hr.	
	Separate values	Mean and S.E.
7·0–6·1	178, 158, 196, 152, 148, 223, 188, 221, 249, 216, 135	187·6 ± 11·1
6·0–5·1	182, 165, 173, 117, 236, 106, 189, 204, 217, 137, 175, 196, 222, 190, 164, 164, 118, 138, 147, 172	170·6 ± 7·8
5·0–4·1	96, 247, 156, 166, 129, 123, 64, 230, 161, 138, 160, 219, 229, 161, 162, 161	162·6 ± 12·4
4·0–3·1	166, 139, 177, 125, 117, 144, 161, 144, 81, 147, 179, 231, 148, 139, 131, 163, 160, 122, 217, 213, 114, 195, 119	153·6 ± 7·6
3·0–2·1	159, 180, 114, 155, 155, 156, 181, 204, 164, 244, 114, 120, 110, 102, 169, 163	155·6 ± 9·4
2·0–1·1	139, 110, 110, 120, 121, 117, 135, 201, 99, 111, 116, 161, 125	128·0 ± 7·5
1·0–0	128, 42, 35, 86, 36, 67, 76, 87	69·6

Table 2. *Oxygen consumption of* Chironomus *at* 17° C. *at various concentrations of dissolved oxygen. First series: Carbon monoxide-treated animals*

Oxygen concentration c.c./l.	Oxygen consumption cu. mm./g. (wet)/hr.	
	Separate values	Mean and S.E.
7·0–6·1	239, 185, 154, 111, 111, 123, 160, 150, 251, 188, 166	167·1 ± 14·0
6·0–5·1	137, 149, 204, 164, 182, 174, 160, 164, 163, 204, 292, 135	177·3 ± 12·2
5·0–4·1	184, 128, 126, 120, 238, 143, 159, 171, 140	156·5 ± 12·4
4·0–3·1	162, 119, 181, 110, 134, 110, 150, 143, 225, 164, 122	147·3 ± 10·5
3·0–2·1	139, 112, 125, 99, 118, 132, 106, 126, 128, 130	121·5 ± 4·0
2·0–1·1	68, 89, 75, 75, 80, 56	73·8 ± 4·6
1·0–0	45, 39, 22	35·3

by 1 c.c. oxygen per litre. The oxygen concentration corresponding to each respiratory rate is taken as the mean of its value at the beginning and at the end of the time interval in question. It will be seen from Fig. 2 that from air

saturation down to 3·5 c.c. oxygen per litre there is no difference between normal and CO animals; but below 3·5 c.c. per litre the normal animals consume more oxygen than the CO animals. The differences between the two pairs of points between 3 and 1 c.c. per litre are statistically significant. Nevertheless, this series of experiments could not be regarded as conclusive, since there was a size difference in the larvae used in the two series of experiments. The average weight of the untreated animals was 12·9 mg. and of the CO-treated animals 15·7 mg.

The correlation coefficient, r, between weight and respiratory rate is found to have a value of $-0{\cdot}3$ for those readings at 7·0–6·1 c.c. per litre and $-0{\cdot}4$ at 6·0–·1 c.c. per litre. From Simpson and Roe's Table IX (1939, p. 236) it can be seen that these values of r are not significant. It is therefore improbable that the difference found between the two curves of Fig. 2 is due to the size differences between

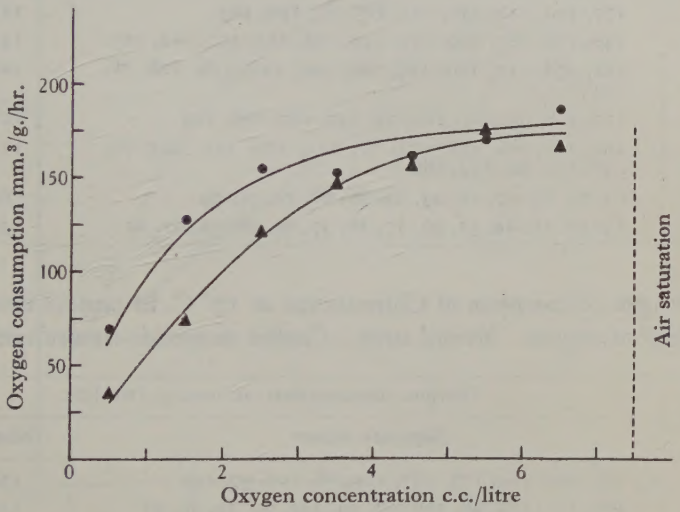

Fig. 2. Rates of oxygen consumption at 17° C. of *Chironomus* at various concentrations of dissolved oxygen; ● normal animals, ▲ animals with carboxyhaemoglobin. Data from Tables 1 and 2.

created and untreated animals rather than to elimination of the haemoglobin. This is further supported by the fact that the CO animals consume the same amount of oxygen at air pressure as do the normal animals.

Another possible cause of the difference between the two curves of Fig. 2 lies in the fact that the material was found to contain two species. Since the experiments on normal larvae were all done before those with CO animals, any change in the relative frequencies of the two species in the material used might account for the difference between the two curves.

A second series of experiments was therefore done covering the critical part of the curve (3·0 c.c. per litre down to 0·5 c.c. per litre) taking care to eliminate these sources of error. The experiments were carried out in pairs, one series of measurements being made with normal animals, the other simultaneously with CO animals.

The larvae in the two cases had had exactly the same previous history, they were selected so as to be of the same size, and if the material used included both species then equal numbers of each were used in the two series of experiments. The average weights of the animals were 20·5 mg. for the normal and 19·3 mg. for the CO experiments. The difference is slight, and the CO animals are the smaller, so that any influence of size on oxygen consumption would tend to diminish rather than to exaggerate the differences previously found.

Table 3. *Oxygen consumption of* Chironomus *at* 17° C. *at various concentrations of dissolved oxygen. Second series: Untreated animals*

Oxygen concentration c.c./l.	Oxygen consumption cu. mm./g. (wet)/hr.	
	Separate values	Mean and S.E.
3·5–3·1	177, 130, 116, 130, 101, 165, 87, 150, 123	131·0
3·0–2·6	148, 136, 187, 130, 121, 154, 182, 113, 172, 112, 107	142·0 ± 8·7
2·5–2·1	152, 158, 110, 120, 124, 180, 149, 113, 138, 108, 78, 122	129·3 ± 7·9
2·0–1·6	119, 118, 95, 141, 112, 71, 147, 107, 146, 113	116·9 ± 7·5
1·5–1·1	142, 128, 82, 138, 123, 86, 111, 120, 111, 125, 69, 97, 135, 64, 142, 166	114·9 ± 7·2
1·0–0·6	67, 73, 63, 47, 56, 43, 82, 56, 66, 74, 71, 79	64·7 ± 3·5
0·5–0	15, 59, 49, 24, 51, 36, 17, 26, 37, 62, 50, 22, 61, 22	37·9

Table 4. *Oxygen consumption of* Chironomus *at* 17° C. *at various concentrations of dissolved oxygen. Second series: Carbon monoxide-treated animals*

Oxygen concentration c.c./l.	Oxygen consumption cu. mm./g. (wet)/hr.	
	Separate values	Mean and S.E.
3·5–3·1	156, 127, 164, 177, 137, 124, 88, 109, 97, 149	132·8
3·0–2·6	100, 151, 180, 96, 166, 97, 79, 147, 94, 73, 79, 91	112·7 ± 10·8
2·5–2·1	100, 91, 110, 109, 71, 103, 101, 81, 75, 125, 102, 147, 98, 50, 101, 122, 79	98·0 ± 5·5
2·0–1·6	76, 116, 78, 127, 127, 89, 126, 85, 72, 61, 79, 85, 29, 72	87·3 ± 7·5
1·5–1·1	88, 91, 88, 96, 71, 59, 63, 85, 58, 51, 60, 32, 78, 55	69·6 ± 5·0
1·0–0·6	34, 38, 52, 71, 57, 49, 59, 46, 63, 50, 50, 53, 33, 45, 63, 32, 52, 59, 44	50·0 ± 2·5

In this series of experiments the readings are grouped into classes differing by 0·5 c.c. per litre. The results are given in Tables 3 and 4 and Fig. 3. It will be seen that the curves differ very little from those of Fig. 2, except that the maximum rate of respiration is lower. This may possibly be due to the fact that the larvae used in this second series of experiments were larger than those used in the first series. The differences between the pairs of points for normal and CO animals are statistically significant from 2·5 c.c. per litre down to 0·6 c.c. per litre; at 2·6–3·0 c.c. per litre the significance of the difference is doubtful.

In Fig. 3 the difference between the curves obtained for normal and for CO animals is also plotted, giving the amount of oxygen carried by the haemoglobin at various oxygen concentrations. The maximum amount of oxygen is carried by the pigment at an oxygen concentration of about 1·5 c.c. per litre; this corresponds to about 32 % of the total consumed at that oxygen concentration.

Since there exist species of *Chironomus* which possess no haemoglobin it would be possible to use these as controls to test whether the concentration of carbon monoxide used has any effect on respiration other than that of eliminating oxygen carriage by the pigment. The work had to be discontinued before this could be done, but it is unlikely that the amount of carbon monoxide used, which corresponded to

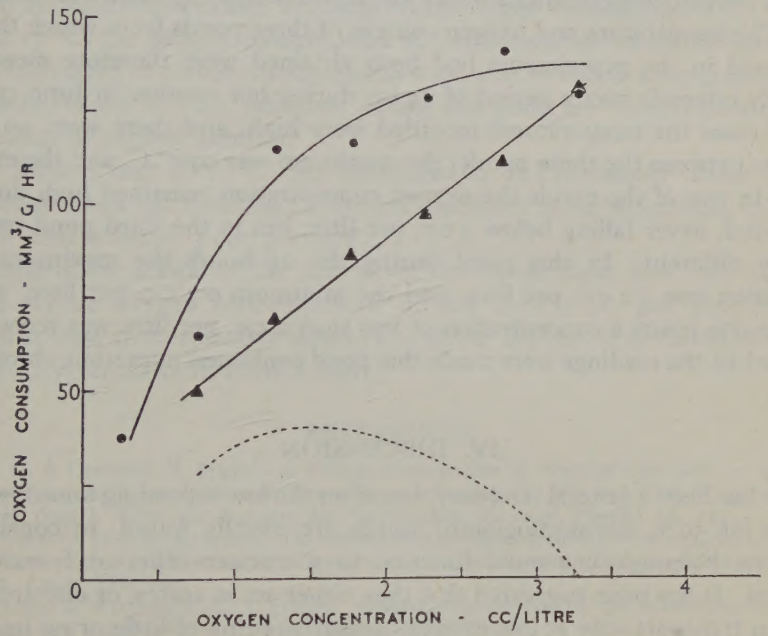

Fig. 3. Rates of oxygen consumption at 17° C. of *Chironomus* at various concentrations of dissolved oxygen; ● normal animals, ▲ animals with carboxyhaemoglobin. The broken line represents the difference between the rates of oxygen consumption of normal animals and those with carboxyhaemoglobin. Data from Tables 3 and 4.

a pressure of 1/5, relative to oxygen, had any effect on cell enzymes. The affinity of haemoglobin for carbon monoxide is high, and therefore a low relative pressure is sufficient to convert the pigment completely to the CO compound. In all organisms so far studied the affinity of oxidase for carbon monoxide is low, and therefore a high relative pressure is necessary before there is any appreciable inhibition of its action. Warburg (1926) found that for yeast at 20° C. that pCO/pO_2 must reach a value of 10 to bring about 50 % inhibition of respiration. Keilin (1927, 1929) confirmed Warburg's findings for oxidase activity in yeast, and also studied the inhibitory action of carbon monoxide on the activity of indophenol oxidase of sheep's heart muscle and of polyphenol oxidase of potato. In the case of heart muscle the inhibitory action of carbon monoxide is slightly greater than it is in yeast; in the potato it

is greater still, pCO/pO_2 of 9 giving 83% inhibition. Wolsky (1938) finds that in *Drosophila melanogaster* pupae pCO/pO_2 of 5·6 gives 50% inhibition. In all these cases relative pressures of carbon monoxide of less than 1 are ineffective. In the present experiments it is therefore very improbable that oxidases were affected by the pressure of carbon monoxide used. This is also borne out by the fact that the CO animals have been found to consume as much oxygen as do untreated animals at oxygen concentrations from air saturation down to approximately 3·5 c.c. per litre: this could hardly be the case if their oxidases had been affected by the carbon monoxide.

It was thought desirable to find out what are the maximum temperatures and the minimum oxygen concentrations which the chironomids experience in natural conditions. The temperature and oxygen content of three ponds from which the chironomids used in the experiments had been obtained were therefore measured at two-hourly intervals over a period of 24 hr. during hot weather in June 1940.[1]

In all cases the temperatures recorded were high, and there were no striking differences between the three ponds: the maximum was 29·0° C. and the minimum 16·0° C. In two of the ponds the oxygen concentration remained high during the whole period, never falling below 4 c.c. per litre, but in the third pond conditions were very different. In this pond during the 24 hours the maximum oxygen concentration was 3·2 c.c. per litre, and the minimum 0·3 c.c. per litre, while for 16 consecutive hours a concentration of less than 2 c.c. per litre was recorded. At the time when the readings were made this pond contained numerous chironomids.

IV. DISCUSSION

There has been a general tendency, based on the low unloading tensions (oxygen pressures for 50% oxyhaemoglobin) which are usually found, to consider that invertebrate haemoglobins must function in a manner different from those of vertebrates. It has been suggested that they either act as stores, or else are adapted for oxygen transport only at low oxygen pressures, being of little or no importance at air pressure. Nevertheless, it is of interest to note that in the only cases so far investigated in which adequate experiments, using the carbon monoxide technique, have been carried out, it has been shown that the pigments, just as in the vertebrates, are functional in oxygen transport at air saturation. This has been shown to be the case in *Tubifex tubifex* by Dausend (1931), in the earthworm by Krüger (1938) and by M. L. Johnson (unpublished, see Fox, 1940), and in *Sabella pavonina* by Ewer & Fox (1940). Most of the work of other authors in this field is rendered inconclusive by their use of high concentrations of carbon monoxide and their failure to consider its possible direct effect on the respiratory enzymes. The present work is therefore of interest in that it demonstrates that in the case of chironomid larvae the range of oxygen concentration over which the pigment is of importance lies well below air pressure, at any rate for animals which have previously been in well

[1] My sincere thanks are due to Mr L. Haig for affording me every hospitality and help in making these readings.

erated water. Harnisch (1936), however, finds that the haemoglobin is functional t air saturation in animals which have been subjected to oxygen lack and are presumably paying off an oxygen debt. No further investigation of this question has been made and the present work deals only with animals which have been kept in conditions where oxygen was abundant.

SUMMARY

1. At 17° C. the oxygen consumption of *Chironomus* larvae shows little diminution when the oxygen concentration in the water decreases from 7·5 c.c. per litre (air saturation) to about 3 c.c. per litre. Below 3 c.c. per litre a further decrease in oxygen concentration causes a falling off in oxygen consumption.

2. At 17° C. the oxygen consumption of *Chironomus* larvae whose haemoglobin has been converted to carboxyhaemoglobin is as great as that of normal animals from air saturation down to 3 c.c. per litre. Below this point the oxygen consumption is less than that of normal animals.

3. It follows that at 17° C. the haemoglobin of *Chironomus* larvae from well aerated water does not function in oxygen transport at air saturation, but only at oxygen pressures below 3 c.c. per litre.

4. Some observations of the habitat of the larvae show that they can live in situations where oxygen concentrations of less than 2 c.c. per litre persist for periods of at least 16 consecutive hours. The larvae are also to be found in situations where the oxygen concentration is high.

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THE INFLUENCE OF VARIOUS PHYSICAL AND BIOLOGICAL FACTORS OF THE ENVIRONMENT ON HONEYBEE ACTIVITY. AN EXAMINATION OF THE RELATIONSHIP BETWEEN ACTIVITY AND SOLAR RADIATION

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I. INTRODUCTION

In attempting to assess the value of the honeybee as a pollinator of fruit and other crops, particularly in a season when inclement weather is likely to reduce the number of wild pollinating insects, it was realized that very little is known of the influence of the physical factors of the environment on honeybee activity. It was therefore decided in the first instance to study the effects of solar radiation, humidity, actual precipitation of water vapour, and temperature on the flying activity of the honeybee. Later it is hoped to continue with a study of the effects of nectar abundance and concentration and also the biological condition of the colony itself upon activity. It should be realized, however, that although it is simplest to study each of these factors separately they are very largely interdependent upon one another and that all work together to influence the final result. In the present paper the influence of solar radiation is considered.

II. DISCUSSION OF PREVIOUS WORK

Various workers have studied this subject, mostly with regard to its influence on honeybees at that time of the year when they are required for orchard pollination.

Hutson (1926) states that sunlight favourably affects flight but does not urge bees to leave the hive if other conditions are unfavourable. Phillips (1930) points out that sunshine is not necessary for flight, but cloudy weather tends to keep the bees near the hive by confining them to short flights. Marshall *et al.* (1929) state that bees are most active on bright, warm days. These statements are without doubt in conformity with general observations made by many beekeepers; they do not, however, allow any exact determination of the importance of light intensity on honeybee activity. Brittain *et al.* (1933) made very much more detailed studies of the effect of the intensity of white light, and also various wave-lengths of light on bee activity. They point out that although sunlight is an important factor in influencing the activity of bees, it alone will not cause them to work if the tem

erature is too low. They also state that an interesting effect of lack of sunlight can e observed on shaded limbs of apple trees, the flowers of which are not pollinated anything like the same extent as those on limbs exposed to sunlight. Their counts ade of the number of bees present on apple flowers at different times of the day now that, even at optimum temperatures, fewer bees were present in hazy weather, en without definite cloud banks, than when the sky was clear. They claimed to now that there is a general trend upward of bee activity, corresponding with creasing light values, and a corresponding decrease when light values normally ll off in the afternoon, or from the effects of clouds, haze or fog at any time during e day. It was further claimed that, within the temperature range of bee activity, ght apparently has a more important influence than slight changes in temperature. hey were unable, however, by this method of sampling (i.e. counting the number f bees present on the flowers) to secure any clear indication that any particular ave-length of light had any greater stimulative effect on bee activity than any her, even though it might have been expected from the work of Bertholf (1931, 931 *a*), who demonstrated the stimulative effect of ultra-violet light on insects, cluding the honeybee. One of Brittain's collaborators, J. M. Cameron, however, aking further observations on this subject during the honeyflow from Golden-rod y means of an electrical counter which determined the number of bees leaving nd entering the hive during quarter-hour periods throughout the day, claims to ave shown that the curve showing the average honeybee flight throughout the ay follows more closely the changes in ultra-violet light values than white-light alues; there being a higher correlation between ultra-violet light values and also hite-light values and honeybee activity, than between temperatures and honeybee ctivity. He calculated simple correlations of the average temperature, intensity f clear light and intensity of ultra-violet light, with number of bees leaving a hive n successive half-hour periods throughout the day, the correlations he gives being or temperature 0·67, for intensity of clear light 0·84, and for the intensity of ultra-iolet light 0·92. He suggests that all three conditions acting together probably ecide the degree of activity as shown by the multiple correlation of 0·93 which he btained and stated that the importance of ultra-violet light is further shown by he 'beta values' calculated. It is unfortunately not apparent what is meant by he latter, but they are probably partial regression coefficients, the units not being pecified.

Unfortunately, when more closely examined the figures given by Brittain and lso by Cameron are found to be not nearly so valuable as would appear at first ight. In the general discussion of the effect of light on bee activity during the pple flowering period it is shown that there is a general trend upwards of bee ctivity corresponding with increasing light values, and a corresponding decrease vhen light readings normally fall off in the afternoon. This is undoubtedly true ut not very informative, since the amplitude of the main periodic variation is in eneral sufficiently large to mask the effect of minor irregularities, and the same esult would hold if light intensity were replaced by any measurement having a iurnal cycle. This point is discussed in greater detail in the next section.

The irregularities in light intensity caused by the passage of clouds are of such frequency that it is necessary to take observations at short intervals in order to show them. The data presented by Brittain are inadequate, as only half-hourly readings are shown. It is also not clear from the test in what way the average bee counts were obtained. Each point is presumably the mean of several counts, since the values are not always integral, but there is no indication whether records of a number of different observers have been combined or whether a mere average of counts for adjacent time intervals has been made.

Visual examination of the four diagrams shown gives so little impression of association between deviations from trends that it does not seem worth while to attempt any analysis of the figures. On account of the long time intervals between readings the value of these records is slight, though the observations of 25 May 1932 do show a very pronounced drop in bee activity associated with the abnormally early fall in light intensity.

The figure given by Cameron representing data collected during the honeyflow from Golden-rod is probably a summary of much more valuable information. Apart, however, from statistical errors, the data are not well presented. The legend describes one curve as 'number of bees × 10'; this is taken in the present paper to mean that the actual number of bees is 10 times the reading of the graph and not one-tenth of this, as the latter interpretation leads to unreasonably low counts. From Cameron's statement that figures for periods earlier than 8.30 a.m. represent the records of 2 days only, it would appear that the graph is drawn from means of a number of day's-observations for each interval, the number of days available possibly varying considerably for different hours of the day. It is stated that 415 observations in all were made, so that they probably extended over at least 10 days. Any such inequality of representation of the different days would be in every way undesirable, as the means for some periods might be seriously biased relative to others. It is questionable, therefore, whether any reliable conclusions may be drawn from the data as presented; this is the more unfortunate as the records may originally have been excellently suited to a study of light effects on bee activity.

III. ANALYSIS OF RESULTS OBTAINED IN PRESENT INVESTIGATIONS AND CONCLUSIONS

On 5 days during the summer of 1940 (12, 19, 26 June, 3 July and 25 September) on all of which temperature and other weather conditions were favourable for bee activity, observations were made throughout the day on the numbers of bees leaving a certain hive. From early morning until evening counts were made in successive $7\frac{1}{2}$ min. periods of all bees leaving the hive. The only one of the 5 days for which the bees were not already active at the beginning of the observations and had ceased flying by the middle of the afternoon was 25 September. In order that only periods of bee activity may be considered, the observations of the early and late hours of this day have been omitted and the time studied reduced from 12 or $12\frac{1}{2}$ hr

$7\frac{1}{2}$. In analysing these counts it seems preferable to work with proportional ather than with absolute changes, and the records have therefore been subjected a logarithmic transformation. The effect of this change of unit is, for example, attach equal importance to a doubling of the number of bees leaving the hive, matter whether the increase is from 10 to 20 or from 100 to 200, and thus simplify the comparison of changes in activity in colonies of different strength. order to avoid difficulty with zero counts, a convention is made that the umber of bees is increased by one before transforming. The index of bee activity any $7\frac{1}{2}$ min. period is thus taken to be the logarithm (to base 10) of 'one plus the umber of bees leaving the hive in that period'.

This measure of activity for successive periods has been considered in regard the intensity of solar radiation as measured by a Callendar electric recorder, tuated at a distance of about 400 yards from the apiary. Though the curve presenting the changes in light intensity throughout any one day possesses many regularities, on account of the frequent passage of clouds, there is generally a ell-marked diurnal cycle showing a maximum in the middle of the day. This was ue of four of the observation days, but 26 June was exceptional in that there curred a prolonged dull period from about 11.45 to 13.00 G.m.t. Now the rate f egress of bees from the hive shows a diurnal cycle of similar character, rising om a very small figure in the early morning to a maximum in the middle of the ay and falling again in the evening. In one sense, then, there is no doubt of the xistence of a positive association between light and bee activity. But this association of little interest, as the same property would hold for any quantity—no matter ow obviously irrelevant—which shows a similar diurnal cycle.

The real importance of the data lies in their answer to the question: 'Are the eviations of the activity rates of the bees from the trend shown by the diurnal ycle associated with similar deviations in the light intensity?' In order to test is and to estimate the magnitude of the effect, it is essential first to eliminate from oth observations the component due to the cycle. Two methods might be adopted. he first is to fit polynomials of sufficiently high order to both records and to onsider only the association of deviations from these polynomials. Tables for aiding is process are given by Fisher & Yates (1938, Table XXIII). It is not immediately lear what order of polynomial should be used in order to eliminate the trend atisfactorily, and more consistent results appear to be obtained with greater implicity by eliminating differences between successive half-hour periods. This s simply accomplished by the technique of the analysis of variance and co-variance Fisher, 1938), treating sets of four observation pairs as 'blocks' and forming omponents of variation within these blocks.

From this analysis may be computed, for each of the five days, a regression oefficient to represent the average increase in activity resulting from a unit increase n the rate of radiation at any time of the day. This regression coefficient leads to n estimation of the proportionate increase in the rate of egress of bees from the ive corresponding to a unit deviation of the rate of radiation from its trend line. he results are summarized in Table 1.

There is no doubt of the significance of the effect of the light intensity. There are considerable differences in the magnitude of the effect on the 5 days, but it is not apparent what the cause may be. The largest value of the regression coefficient occurs on 25 September when the temperature was low and there were very few bees flying. Conditions of activity at that time of the year are, however, scarcely comparable with those of June and July, and for the four earlier days there is some indication that the higher values of the regression coefficient are associated with the brighter days. On the other hand, the mean activity for the day appears to

Table 1. *Summary of observations on light intensity in its effect on bee activity*

Date	12 June	19 June	26 June	3 July	25 Sept.
Duration of observations (G.m.t.)	7.00–19.00	6.00–18.30	6.00–18.30	6.30–19.00	7.30–15.00
No. of observations	96	100	100	100	60
Temp. in ° F. during observations:					
Minimum	58·3	54·0	52·0	57·9	45·5
Maximum	67·6	70·0	65·0	71·0	57·8
Mean	64·5	65·4	60·1	65·1	52·7
Mean radiation rate in cal./sq. cm./min.	0·479	0·743	0·684	0·385	0·483
No. of bees leaving per 7½ min.:					
Minimum	15	23	0	26	0
Maximum	184	200	341	394	24
Mean (arithmetic)	78·6	99·8	97·8	202·8	3·9
*Mean index of bee activity	1·84	1·93	1·84	2·21	0·575
Increase in bee activity per 0·1 cal. increase in radiation rate	0·0425 ±0·0094	0·0771 ±0·0116	0·0689 ±0·0101	0·0452 ±0·0076	0·0856 ±0·0165
% increase in no. of bees per 0·1 cal. increase in radiation rate	10·3	19·4	17·2	11·0	21·8
Correlation coefficient of bee activity and radiation rate	0·47	0·61	0·62	0·57	0·62

* The index of bee activity in each 7½ min. period is taken to be the logarithm (to base 10) of 'one plus the number of bees leaving the hive in that period'.

bear little relationship to the mean rate of radiation. Within any one day an average increase of about 15% in the number of bees leaving the hive may be expected when the radiation rate increases by 0·1 cal./sq. cm./min. Approximately 30% of the variability of bee activity about its trend may be ascribed to irregularities in light intensity.

It would in theory be possible to make an exactly similar analysis of the counts using temperature records instead of light intensities, or alternatively obtaining regressions on both observations. The temperature is, however, to the order of accuracy of its measurement, much too regular in its changes throughout the day and the elimination of components ascribable to its diurnal cycle would leave little information for a study of its effects on bee activity.

In spite of the criticisms which have been made earlier of the presentation of Cameron's data, it seemed of interest to attempt a new analysis of them by the technique used for the records now under discussion. For this purpose the possible inequalities in the presentation of days in the means were neglected, and the mean counts and light intensities read with two-figure accuracy from the graph. Between 8.30 a.m. and 6.15 p.m. thirty-nine pairs of records for successive quarter-hour

riods were obtained, and the trends removed by eliminating differences between s of three. Again a logarithmic transformation was employed.

The results of this analysis cannot, unfortunately, be put in a form directly mparable with those of Table 1, as Cameron does not state his units of light ensity. In terms of the scale units actually employed by Cameron for his light asurements, without correcting for the factor given in the legend, the partial gression coefficients of bee activity on clear and on ultra-violet light are 0072 ± 0·0030 and −0·0020 ± 0·0024. There is no indication that ultra-violet ht has any significant effect other than the component due to its correlation with ar light, and the regression coefficient on clear light alone, 0·0049 ± 0·0014, fficiently represents the situation. The mean intensities of clear and ultra-violet ht over the periods studied were 48·2 and 54·3 and the mean index of activity of e bees 2·35 for a quarter-hour period. The mean number of bees leaving the ve was 239 per quarter hour. The regression coefficient shows an average increase 1·1 % in the number of bees leaving the hive for a rise of one unit in the rate radiation of clear light. The correlation coefficient between clear-light intensity d bee activity after the elimination of trends, 0·57, shows about 32 % of the riations in activity to be accounted for by variations in radiation. The mean mperature rose from 67° F. at 8.30 a.m. to 73° F. at 1.00 p.m., and afterwards ll steadily to 65° F. at 6.15 p.m. As with the other data, its trend was too regular r information to be obtained on its effects on activity.

It seems, then, that Cameron's conclusions as to the relative importance of clear d ultra-violet light in determining bee activity must be rejected. The reversal the situation appearing in his figures is, of course, little dependent on the use a logarithmic transformation of the data and almost entirely due to the elimination the effects of the diurnal cycle. Unfortunately it is impossible to reconstruct and -analyse the full 415 observations mentioned by Cameron, but, assuming that his eans for different periods of the day have not been unduly distorted by non-thogonal representation of the different days, it appears that deviations of clear-ght intensity from its trend are directly reflected in the activity of the bees, but at ultra-violet light is of little importance in this respect apart from the correlation its measurements with those of clear light.

IV. SUMMARY

A review of the literature shows that those who have studied the influence of olar radiation on honeybee activity are agreed that it is an important limiting ctor. This is in general agreement with observations made by beekeepers.

Brittain *et al.* (1933) give a detailed account of the effects of solar radiation on oneybee activity, and claim that, as might be expected from the work of Bertholf 931, 1931*a*), a higher correlation between activity and the intensity of ultra-violet adiation exists than between activity and radiation of clear light. However, fresh nalysis of their data throws considerable doubt on their conclusions.

New data were collected which clearly show an association between variations n honeybee activity and the radiation of clear light.

We wish to express our thanks to Dr C. B. Williams and the members of th Bee Department for their interest and assistance in this investigation.

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THE ACTION OF POTASSIUM ON ECHINODERM, MOLLUSCAN AND CRUSTACEAN MUSCLE

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(With Six Text-figures)

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ME years ago, the writer published an account of the action of potassium on the ops of *Aplysia* and *Helix*, and the hearts of *Maia* and *Homarus* (Wells, 1928). was pointed out that the preparations used show a general resemblance in their sponses to changes in potassium concentration, although they include plain and iped muscle, and are taken from molluscs and crustaceans. Since that time he s made similar experiments on other preparations. The results are now described d discussed from a comparative standpoint. It appears that potassium—in ntrast to calcium, which has the most diverse effects—produces essentially milar responses in muscles from animals of various different phyla, and of quite fferent histological and functional nature. The suggestion is made that all types rhythmic muscle are essentially alike in their responses to comparable changes the potassium concentration of the environment.

MATERIAL AND METHODS

The cloacal complex of Cucumaria elongata

As far as the writer is aware, no kymograph records of a rhythmic preparation om an echinoderm have yet been published. Crozier (1916) described the actions various ions on the cloacal complex of a large Bermudan holothurian, but did not se the kymographic method. The writer finds that a fairly satisfactory preparation an be made by excising the cloacal complex of *Cucumaria elongata* (i.e. the gut all and the hinder end of the body-wall musculature, the whole being slit up ngitudinally to allow ready penetration of the bathing fluid) and suspending it, a longitudinal strip, in a bath of suitable design (e.g. Wells, 1937, Fig. 4).

The hearts of Aplysia punctata *and* Helix pomatia

Papers on the potassium relations of these hearts are already available (see leymans (1923) for *Aplysia*, and Lovatt Evans (1912), Hogben (1925) and Cardot 1922) for *Helix*). In no case, however, have the experiments been conducted in

such a way as to allow ready comparison with those made by the writer on other species, and he has therefore reinvestigated the question in order to make such comparison possible. The hearts were perfused by means of a constant-pressure cannula (Hogben, 1925, Fig. 2) inserted into the auricle. In the case of *Helix* there exists a difference of opinion between Lovatt Evans, who states that the heart is peculiarly insensitive to potassium, and Hogben and Cardot, who deny this. The writer's experiments lead to the latter conclusion. Lovatt Evans does not explicitly state in his paper that the solutions of abnormal potassium content were applied to the interior surfaces of the heart. If they were not, then insensitivity would be expected.

The heart of Carcinus maenas

Descriptions of the ion relations of various decapod hearts are already available (Hogben, 1925; Wells, 1928; Zoond & Slome, 1929). The different species vary, as described below, in their reaction to moderate potassium excess, and it was felt that further data were desirable. The heart of the shore crab has not yet been studied from this standpoint, and was therefore chosen for the present work. It was perfused by means of a constant-pressure cannula inserted into the sternal artery.

Solutions

Bathing and perfusion fluids were made by mixing isotonic solutions of the single salts—0·6 *M* NaCl and KCl, and 0·4 *M* $CaCl_2$ and $MgCl_2$ for the marine species, whose body fluids are normally isotonic with sea water, and 0·125 *M* NaCl and KCl, and 0·083 *M* $CaCl_2$ for *Helix*, whose blood is roughly isotonic with frog Ringer.

In each experiment two fluids were employed. One consisted of NaCl, to which appropriate amounts of the divalent metal chlorides had been added, and the other of KCl, with identical additions. A trace of buffer was also included, and the *p*H was adjusted to 7·4 for *Aplysia*, or 8·0 for the other three species. These two fluids were mixed in various proportions, and the mixtures were applied to the tissue. In this way, the ratio of Na to K was varied at will, while the osmotic pressure, *p*H, and calcium and magnesium concentrations remained constant. The details of these concentrations are given in the figure legends; for *Cucumaria*, the calcium and magnesium were present in the same amount as in sea water, while the various hearts were studied in magnesium-free mixtures.

Plan of experiments

Ideally, each experiment should start in a 'normal' mixture, whose potassium content is equal to that of the body fluids. In the case of many invertebrates however, the potassium content of the blood is not known. As most preparations will remain active for some time in the absence of potassium, strictly comparable results can be got by taking a potassium-free mixture as the starting point in each

se, and studying the effects of changing from this to a series of solutions with fferent potassium contents, returning to the potassium-free fluid after each posure. This was the method adopted in the experiments here described.

GENERAL DESCRIPTION OF THE POTASSIUM EFFECTS

'Low potassium' effect

The general picture given by a rhythmic muscle in response to sudden change m a potassium-free fluid to one containing a small amount of potassium (say a: K = 100: 1 or 2) is as follows:

(1) Immediately after the change there is a sharp drop in tone.

(2) The change is followed by a more or less well-defined period of inhibition otassium paradox' of Libbrecht (1921)).

(3) After the paradox, the beat is more vigorous, more regular, and above all re lasting than before.

On changing back to potassium-free, the most striking result is a sharp tone e. There is no paradox.

g. 1. *Cucumaria* cloacal complex. 3·5 c.c. $CaCl_2$ and 17·5 c.c. $MgCl_2$ to 100 c.c. NaCl (or KCl). ecord begins in potassium-free fluid. First signal, Na: K = 100: 2. Second signal, return to potassium-ee. Third signal, Na: K = 100: 10. Fourth signal, return to potassium-free.
In all records: read from left to right; time signal marks once a minute.

The tone drop caused by low potassium concentrations is shown by all the eparations employed in this research (Figs. 1–4). It appears in a very wide range other rhythmic preparations, and perhaps in all. It is shown, for instance, by ne gastropod and insect crops, by the earthworm gut, and by vertebrate plain and ardiac muscle. The chief differences in appearance between records from different uscles result from variation in the relative amplitudes of the tone and rhythm ntractions. In hearts, and especially in crustacean and vertebrate hearts, the tone anges are relatively small, while in gut preparations they are generally very great.

The tone rise seen on returning to a potassium-free fluid is simply the reciprocal this drop, and is also of general occurrence.

The potassium paradox is usually shown by the *Cucumaria* preparation, but as not clearly seen in the records of the molluscan hearts, and only occasionally ppeared in the case of *Carcinus*. It is, however, a widely distributed phenomenon. t has been reported as occurring in the *Helix* and *Dytiscus* crops (Wells, 1928;

Hobson, 1928), in the *Limulus* heart (Chao, 1934), in the *Maia* and *Homarus* heart (Wells, 1928), and in vertebrate plain and cardiac muscle (Libbrecht, 1921 Jendrassik, 1924). The diastolic standstill produced by small amounts of potassium in the hearts of *Palinurus* and *Octopus* (Zoond & Slome, 1929) is apparently a par

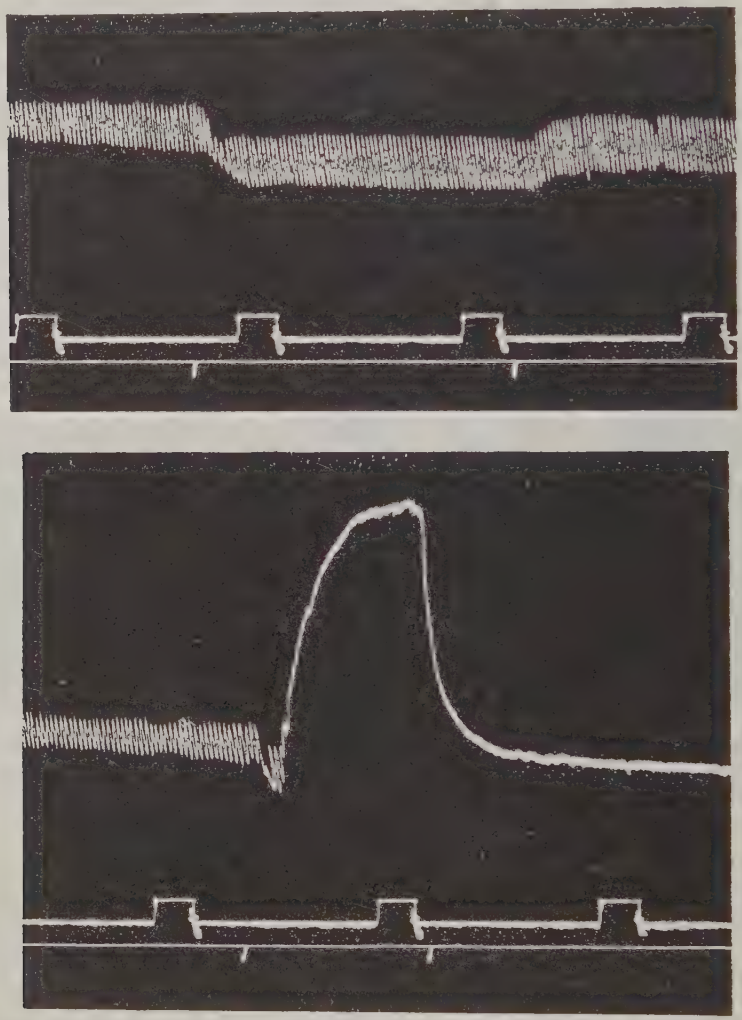


Fig. 2. *Aplysia* heart. 4 c.c. $CaCl_2$ to 100 c.c. NaCl (or KCl). Each record begins, and ends, i potassium-free fluid, and shows, between the signals, the effect of Na:K = 100:1 (above) 100:10 (below).

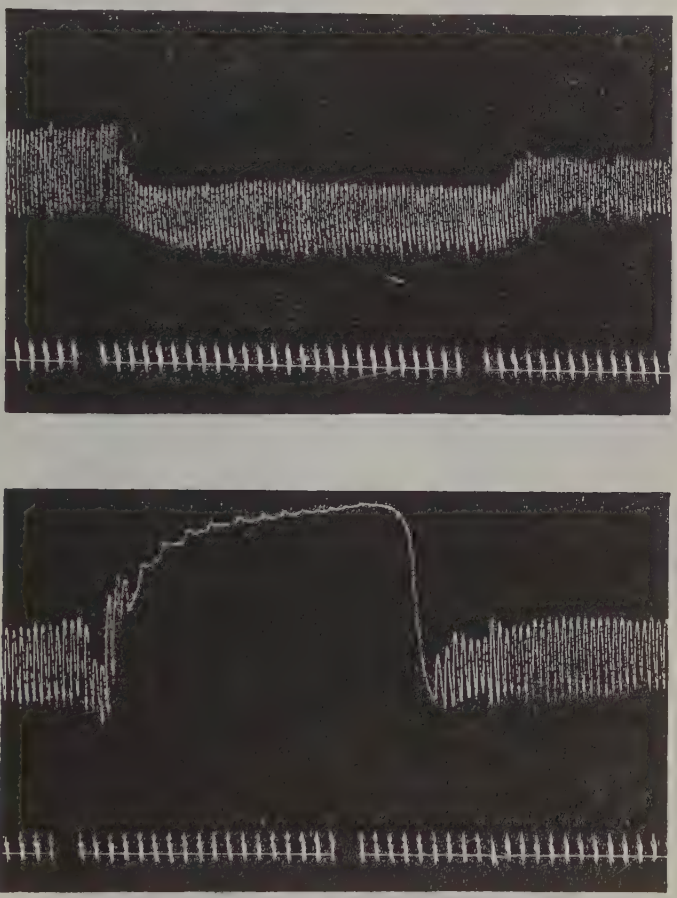
ticularly striking case of potassium paradox. The conditions governing the exter of the paradox are sometimes rather tricky (Chao, 1934). In the *Aplysia* cro appearance of the paradox is favoured by the presence of magnesium or by a hig calcium content (Wells, 1928). The phenomenon could probably be demonstrate in the gastropod heart in suitably designed experiments, e.g. in the presence magnesium.

'High potassium' effect

On changing from a potassium-free fluid to one containing a moderately high tassium concentration (say Na:K = 100:10 or 20), the following responses seen:

(1) In most cases, the preparation passes into contracture.

(2) The rhythmic contractions are inhibited.



g. 3. *Helix* heart. 15 c.c. $CaCl_2$ to 100 c.c. NaCl (or KCl). Each record begins, and ends, in tassium-free fluid, and shows, between the signal breaks in the time trace, the effect of Na:K = 100:2 ove) or 100:20 (below).

On returning to potassium-free, the contracture passes off rapidly, while recovery the beat is less prompt.

Contracture is clearly shown by the *Cucumaria* preparation, and by the snail d sea-hare hearts (Figs. 1–3). It is, in fact, exceedingly widespread among ythmic muscles, and appears to be the most general response to potassium excess.

There exists, however, a group of preparations in which the reaction to moderat excess is somewhat different. After a more or less noticeable transient contracture they reach a level intermediate between the original diastolic and systolic levels and usually nearer to the former. After this the lever traces a horizontal or slowl rising line. This type of response is shown by the frog heart, and by most crustacea hearts (Fig. 4). An intermediate condition appears in the earthworm gut, wher

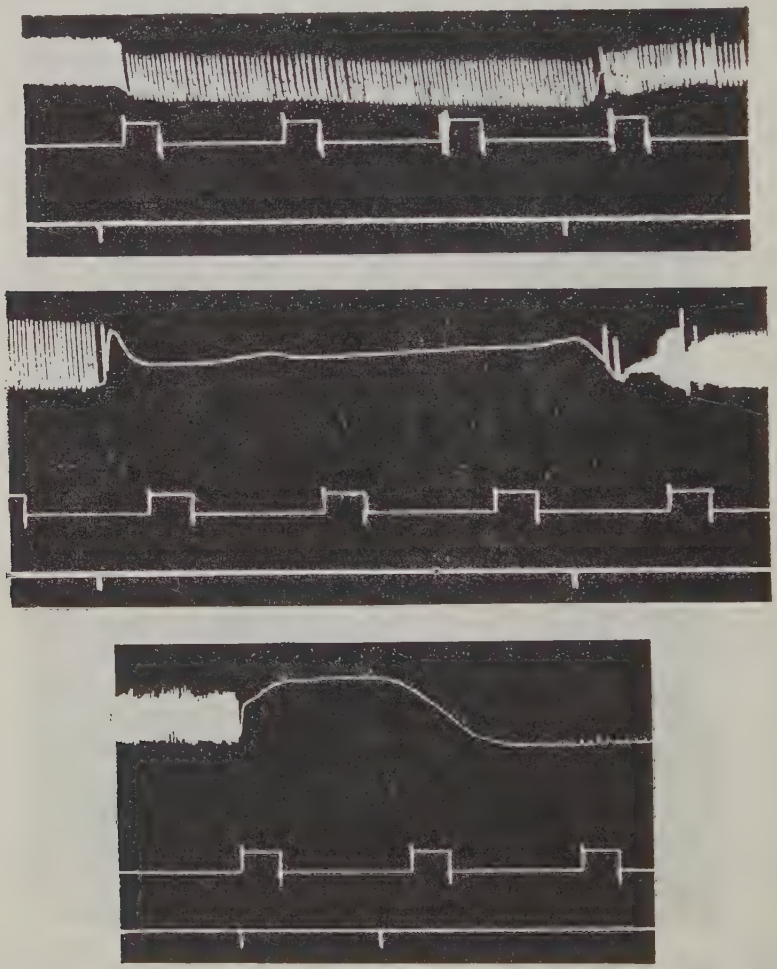


Fig. 4. *Carcinus* heart. 3·5 c.c. $CaCl_2$ to 100 c.c. NaCl (or KCl). Each record begins, and ends, potassium-free fluid, and shows, between the signals, the effect of Na:K = 100:1 (above), 100: (middle) or 100:100 (below).

contracture is evoked, but passes off fairly rapidly (Wu, 1939). The writer do not believe that this variation in reaction pattern indicates a fundamental differen in the physiological make-up of the preparations. In the Crustacea, at least, t heart can be brought to full contracture by sufficiently increasing the potassiu concentration (Fig. 4), and in the spider crab some individuals show prolong

ntracture, and others a response like that in the middle record of Fig. 4, to derate potassium excess (Wells, 1928). A somewhat similar situation is en- ntered in vertebrate skeletal muscle. Even in different muscles of the same mal, the type of response to potassium excess varies greatly. Thus the frog's fibularis consists of two parts, one of which shows great and prolonged potassium ntracture, while the other responds to identical potassium concentrations with ight, transient, fibrillating contraction (Sommerkamp, 1928). The records show ather striking resemblance to those got from rhythmic muscles—the 'Tonus- ndel' of the ileofibularis to a snail heart, and the other portion to a lobster art—and it is at least possible that in rhythmic muscles we are concerned with hysiological differentiation of the same kind.

Inhibition of the rhythm is always seen with sufficient potassium excess.[1] Many hors (e.g. Heilbrunn, 1937; Fenn, 1940) refer to a stoppage of the type seen the lower parts of Figs. 2 and 3 as systolic, and to one like the centre part of g. 4 as diastolic. It will, however, be obvious, from an inspection of the accom- ying records, that the heights at which arrest occurs have nothing to do with normal systolic and diastolic heights. Indeed, the figures suggest that the ppage is always diastolic, as regards the rhythm, but superposed on a tonic ntraction of variable pattern. The fact that there are two types of contraction, thmic and tonic, is clearly seen in the records of 'low potassium' effects. casionally, one encounters a heart whose rhythmic mechanism is out of action; h a preparation will trace a horizontal line in potassium-free with a sharp little p on changing to low potassium, and a rise on returning. In a normal heart, se effects appear as variations of the diastolic level. In describing the reactions high potassium, it is best to restrict the terms systolic and diastolic to the thmic type of contraction. In this sense stoppage is probably always diastolic.

CALCIUM : POTASSIUM ANTAGONISM IN THE *HELIX* HEART

Comparison of the *Aplysia* and *Helix* hearts is interesting. The osmotic pressure the perfusion fluid is nearly five times greater in the former case than in the ter. The general picture of their potassium relations is, however, identical, ovided that the potassium concentrations are given relatively to the concentrations other ions. Broadly speaking, any mixture which exerts a given effect on the *plysia* heart will exert the same effect on *Helix*, if it is diluted five times. The tor which determines whether any given concentration of potassium will evoke e 'low' or 'high' type of response is therefore not the absolute value of that ncentration, but the ratio which it bears to some other variable—either to the ner external ion concentrations, or to some constituent of the cells. This is of urse only a special case of a general rule. Most, or all, rhythmic preparations ve essentially similar potassium responses, when the amount of potassium is

[1] It is assumed throughout this paper that the divalent ion concentrations are kept constant. e fore-gut of *Dytiscus* remains active at very high potassium concentrations if the calcium is also atly increased (Hobson, 1928).

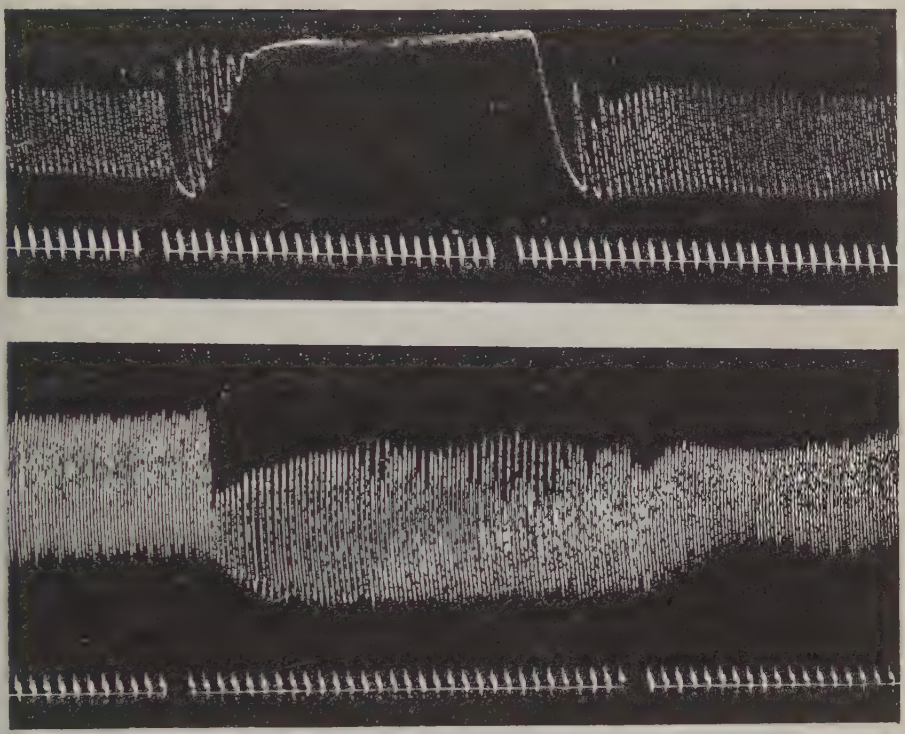
given in proportion to the other ionic constituents of the medium, whether the are taken from marine, fresh-water or terrestrial animals.

A number of experiments were made on the *Helix* heart, to find out whether calcium:potassium antagonism would yield an explanation of this rule. The *Helix* heart is tolerant of a wide range of calcium concentrations. The action of various potassium concentrations was tested, in some experiments with 2, and in others with 15, c.c. of $CaCl_2$ to 100 c.c. of NaCl (or KCl). It was found that the calcium concentration has a marked influence on the amount of potassium required to produce the 'high potassium' type of effect (Fig. 5). In the presence of 2 c.c. $CaCl_2$ contracture is produced occasionally by 7·5 and always by 10 c.c. KCl to 100 c.c. NaCl. With 15 c.c. $CaCl_2$, on the other hand, the effectiveness of potassium seems to be roughly halved, for the 'high' effect is given occasionally by 15 and always by 20 c.c. KCl to 100 c.c. NaCl. It will be noticed, in Fig. 5, that this antagonistic action of calcium extends to both the tone effect and the rhythm effect of potassium.

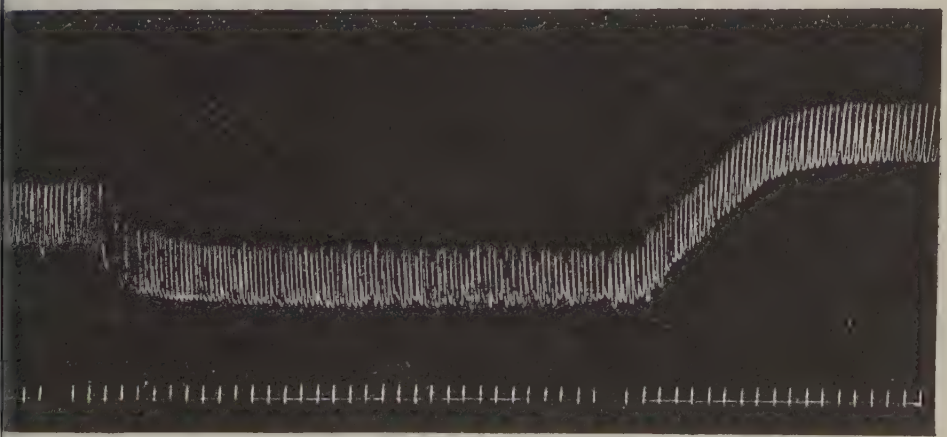
Although these experiments indicate that calcium:potassium antagonism is a powerful factor under these particular conditions, they also suggest that other factors contribute to the difference between the two molluscan species, for increasing the calcium concentration seven and a half times has only halved the effectiveness of potassium.

AMMONIUM:POTASSIUM PARALLELISM IN THE *HELIX* HEART

In the *Aplysia* crop, the ammonium ion closely parallels the potassium ion in its physiological actions. Starting from a potassium-free fluid, a small amount of ammonium chloride produces relaxation of tone and improvement in the rhythm, while a large amount causes contracture (Wells, 1928). Physiological parallelism between the two ions is to be expected, for they are very similar in their physical properties. On the other hand, ammonium chloride solutions contain traces of ammonia, even at only weakly alkaline reactions, and this substance, because of its ready entry into the cells, might exert disturbing actions. In the *Aplysia* crop the amount of ammonium necessary to produce the 'high potassium' effect is about five times as great as the corresponding amount of potassium. Comparable experiments were made on the *Helix* heart. The 'low potassium' effect is easy to produce with ammonium instead of potassium (Fig. 6). The 'high potassium' effect, on the other hand, is not evoked, even when half the sodium is replaced by ammonium. The effect of this substitution is to produce a 'low potassium' response, followed by the gradual onset of a contracture; the latter differs from potassium contracture in its slowness, and in the fact that it endures long after return to ammonium-free fluid. The parallelism between the two ions is therefore less perfect in the *Helix* heart than in the *Aplysia* crop.



5. *Helix* heart. Each record begins, and ends, in potassium-free fluid, and shows, between the al breaks, the effect of Na : K = 100 : 10. Amount of $CaCl_2$, to 100 c.c. NaCl (or KCl), is 2 c.c. ne upper record, and 15 c.c. in the lower.



6. *Helix* heart. No potassium. 2 c.c. $CaCl_2$ to 100 c.c. NaCl (or NH_4Cl). Record begins, and s, in ammonium-free fluid, and shows, between the signal breaks, the effect of Na : NH_4 = 100 : 10.

SUMMARY

1. The effects of various potassium concentrations on the cloacal complex o *Cucumaria elongata*, and on the hearts of *Aplysia punctata*, *Helix pomatia* an *Carcinus maenas* are described.

2. It is pointed out that there exists a fundamental similarity between many and perhaps all, types of rhythmic muscle, as regards their responses to change in potassium concentration.

3. Data on calcium:potassium antagonism and ammonium:potassium paral lelism in the *Helix* heart are presented.

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A COMPARATIVE STUDY OF PERIPHERAL INHIBITION IN DECAPOD CRUSTACEANS

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(With Two Text-figures)

INTRODUCTION

ıL the peripheral muscles of the decapod Crustacea receive, as far as is known, l east one inhibitory axon (Wiersma, 1941). A detailed study of the mechanism this peripheral inhibition has been made for only a few muscles. Marmont Viersma (1938) examined especially the inhibition of the opener muscle of the yfish, and Wiersma & Helfer (1941) the inhibition of the opener and closer scles of the crab, *Cancer antennarius*. Although these studies were limited to so muscles, significant differences in the nature of the inhibitory mechanisms of se preparations were found. The distribution of the inhibitory fibres has since n worked out for species of the *Astacura*, *Palinura*, and *Brachyura*, both for the ıtomical relationships and the functional response to stimulation of the isolated ıibitory fibres (Wiersma, 1941). These recent additions to the knowledge of the tribution of inhibitory axons have made a more extensive comparative study of ıibitory mechanisms possible.

Patterns of innervation. In order to interpret the results of the present in-tigation it will be necessary to review briefly the distribution of efferent fibres the muscles in the distal parts of the leg, as reported by Wiersma (1941). As ı be seen in Fig. 1, innervation of these muscles in the three above-mentioned ɔups is identical with respect to the number and distribution of motor fibres. ıe inhibitory innervation consists always of three nerve fibres which show ferences in distribution between the groups. These distribution patterns of the ıibitors may be briefly described as follows: In all three groups the *opener* ıscle (abductor of dactylopodite) and the *main flexor* (of carpopodite)[1] are ıervated by one inhibitory fibre (I). The second inhibitor (II) innervates the *etcher* (extensor of propodite), and in *Panulirus* and the crabs runs only to this ıscle. In *Cambarus*, however, the stretcher inhibitor also innervates the *closer* ıscle (adductor of dactylopodite). The third inhibitor (III) shows great variation

[1] This has been established with certainty for *Panulirus* only, but is also very likely for *Cambarus* l the crabs.

in its distribution in the three groups. In *Cambarus* it innervates the *bender* (flexor of propodite) and the *extensor* (of carpopodite). In *Panulirus* it innervates the closer, the bender, the extensor, and the *accessory* flexor (of carpopodite).[1] In the crabs the third inhibitor is the 'common' inhibitor of Wiersma (1941), which innervates the opener, the closer, the stretcher, the bender, and the extensor, thus giving the opener and the stretcher a double inhibitory innervation consisting of the respective true inhibitors and the common inhibitor. In the paper mentioned, this way of innervation was studied only in *Cancer anthonyi*, but we found it to be exactly the same in the other species of crabs which have been used in this investigation, and in *Randallia ornata*. Since the latter species belongs to a different superfamily of the Brachyura, it is very likely that all true crabs show the same pattern.

In the following pages, in speaking of inhibition of the stretcher contraction of *Cambarus*, for instance, the term 'stretcher inhibitor-stretcher system' will be used while in the tables roman numerals indicate the inhibitory fibres.

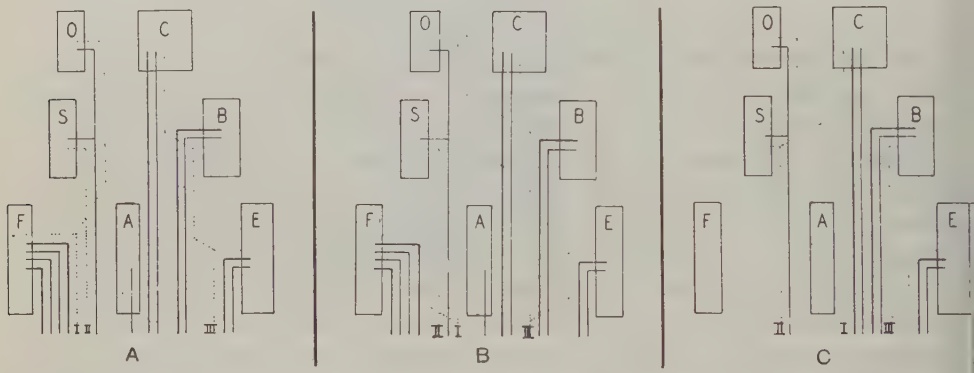

Fig. 1. Scheme of the innervation of the chelipeds and legs. A, *Panulirus*. B, crabs. C, *Cambarus*. Each line represents one axon. Full drawn lines = motor fibres, dotted lines = inhibitory fibres. Notice the similarity in number of the fibres in the different cases and the difference in distribution of the inhibitors. The muscles are represented by the letters: O = opener, C = closer, S = stretcher, B = bender, F = main flexor, A = accessory flexor, and E = extensor.

METHODS

The method of preparing single motor and inhibitory fibres which has been used throughout this investigation has been described in detail for the same preparations which were used here (Wiersma, 1941). In order to study the effectiveness of inhibition essentially the same method was used as that reported by Marmont & Wiersma (1938). In this method the lowest frequency of inhibition which will just suppress any shortening in the muscle when excitation and inhibition are started at the same time is determined for different frequencies of excitation. The necessary stimulating frequencies were obtained with two thyratron stimulators each of which had a range of frequencies more than ample to cover the physiologic range. The stimulators were calibrated and were periodically checked as to frequency in general only small and insignificant variations in the calibrations were found.

[1] This innervation of the accessory flexor was first demonstrated during the present investigation. In the crabs and the crayfish the innervation of this muscle has not yet been found.

The strength of stimulation was always kept low so as to obtain an impulse on h stimulus and yet avoid repetitive discharges. The completeness of the sup- ssion of the contraction was determined by visual observation either with or hout the aid of a binocular microscope, or by recording with an isotonic lever. mparison of the values obtained by these methods showed no appreciable erence.

To study the effect of inhibition of the muscle action potentials an apparatus s used giving series of two shocks at various frequencies. (In the present estigation a frequency of 45 per sec. was usually employed.) The interval ween the two shocks could be varied from 0 to 8 msec. This apparatus has been cribed by Keighley and was used for the study of the potentials of the opener closer of *Cancer anthonyi* (Wiersma & Helfer, 1941).

The animals which were used in the present investigation were, for the Astacura, *mbarus clarkii*; for the Palinura, *Panulirus interruptus*; and for the Brachyura, *ncer anthonyi*, *C. antennarius*, *Pachygrapsus crassipes*, and *Loxorhynchus grandis*.

RESULTS

Determinations have been made for most of the inhibitory innervations as to the effect of frequency of excitation on *Rc* (*Rc* = *Fi*/*Fe*: that is, the ratio between quency of excitation, *Fe*, and frequency of inhibition, *Fi*, necessary just to press the contraction), (B) the *Rc* values of the different systems, and (C) the sence or absence of supplementary inhibition.

A. The effect of frequency of excitation on *Rc*

Marmont & Wiersma (1938) have shown that in the opener of *Cambarus Rc* is arkably constant for different frequencies of excitation. In the slow closer tem, on the other hand, they found that only at low frequencies of excitation uld an *Rc* value be determined, and that at slightly higher *Fe* inhibition was ays incomplete no matter how high *Fi* was made. Most of the systems which e been investigated are less easily inhibited than the opener but more easily ibited than the slow closer of the crayfish. It was found that the majority of preparations in every system showed a remarkable constancy of the ratio at erent frequencies within a certain range. Several examples of this constancy e been listed in Table 1. It should be noted in this table that in the preparation the stretcher system of *Panulirus* the ratio remains constant despite a tenfold rease in *Fe*.

In systems in which *Rc* is high it is invariably found that at higher *Fe* the system not be completely inhibited with any *Fi*. Inhibition thus becomes ineffective ove a certain frequency of excitation. These systems thus resemble the slow closer the crayfish, but none other shows such an extreme case. In these systems it is ier, therefore, to find how this change in *Rc* is established. Of the two possibilities, a gradual increase of *Rc* with an increase in *Fe*, or (*b*) a sudden change to a e in which inhibition is only partial, the latter is the one which is found. metimes a small range of *Fe* in which an increase in *Rc* seems to be present is

Table 1. *Constancy of Rc with changes in frequency of excitation*

Animal	Inhibitor	Excitor	*Fi*	*Fe*	*Rc*
Cancer anthonyi	I and II	Stretcher-opener (both contractions)	12	20	0·60
			15	25	0·60
			18	30	0·60
			27	45	0·60
			37	60	0·62
			60	100	0·60
Panulirus	II	Stretcher	11	20	0·55
			16	30	0·53
			26	45	0·56
			35	60	0·58
			55	100	0·55
			105	200	0·53
	III	Fast bender	36	30	1·20
			55	45	1·22
			70	60	1·17
			119	100	1·19
Pachygrapsus	III	Slow bender	15	45	0·33
			20	60	0·33
			35	100	0·35
			40	120	0·33

Table 2. *Effect of different frequencies of excitation on Rc in cases in which inhibition becomes incomplete at high frequencies of excitation*

Animal	Inhibitor	Excitor	*Fi*	*Fe*	*Rc*
Pachygrapsus	III	Opener	55	45	1·22
			75	60	1·25
			—*	100	—
			75	60	1·25
Panulirus	III	Fast closer	60	45	1·33
			79	60	1·32
			—*	100	—
			80	60	1·33

* *Fi* 180 did not suppress *Fe* 100, but completely suppressed *Fe* 60.

Table 3. *Effect of different frequencies of excitation on Rc in cases in which Rc becomes lower at higher frequencies of excitation*

Animal	Inhibitor	Excitor	*Fi*	*Fe*	*Rc*
Cancer antennarius	I	Opener	17	30	0·57
			26	45	0·58
			35	60	0·58
			55	100	0·55
			62	120	0·51
			110	200	0·55
			60	300	0·20
Cancer antennarius	II	Stretcher	16	30	0·53
			25	45	0·57
			37	60	0·61
			50	100	0·50
			60	120	0·50
			100	200	0·50
			80	300	0·26

riced, but this range is usually very limited in its extent. Thus, if *Fe* values are .en which are not too close together one will show the *Rc* which is constant, while higher frequency cannot be inhibited (Table 2). The frequency of excitation which inhibition in such systems becomes incomplete differs greatly. For example, slow closer of *Cambarus* ceases to be completely inhibitable at a *Fe* of about per sec.; for most of the fast closers the frequency is about 80–100; and in some parations of slow systems the contractions fail to be completely suppressed at *Fe* of about 200 per sec. That fatigue of inhibition can have an influence on these enomena is shown by the fact that on repetition at one *Fe* a contraction may ow at first the normal *Rc*, then show a state of only partial inhibitability, and, after t, again show the normal ratio. At the higher *Fe*'s, however, even a completely sh preparation will be only partially inhibitable. In these cases *Rc* cannot be plied to the incompletely inhibited states as it is by definition 'just complete' ibition.

Some systems show an entirely different picture. In these, instead of being t partially inhibited at high *Fe*, *Rc* drops suddenly to a much lower value. This urs especially in systems in which *Rc* is normally quite low (see Table 3). It uld be noted that in both cases the *Fi* which completely suppresses the traction at *Fe* 300 is even lower than the value which was necessary at 200.

Whereas the *Rc* of the large majority of all the preparations of the different tems was quite constant with frequency, there were a very few scattered pre- rations in which a definite increase in ratio took place and in some others a rease. It is apparent that these phenomena are associated with some particular perty of the individual preparation and are not of general occurrence. We have refore omitted values of this sort from further consideration, and whenever h a preparation was encountered the values were considered not trustworthy d were discarded.

It can thus be concluded that frequency has no influence on *Rc*. This does not an, however, that *Rc* is constant in every system, but that such inconstancies as ur are caused by factors other than the frequency of excitation. In the deter- nation of the *Rc* of different systems these factors form a disturbing influence, will be clear from the following paragraphs.

B. The *Rc* values of the different systems

In respect to constancy of *Rc* in one preparation of one system and of the same stem in different preparations, the systems fall into three groups: (*a*) In a number ry little variation is found in different determinations. (*b*) The second group is mposed of those which show a rather widespread variation both within the same eparation and between different preparations. (*c*) A third group shows two stinct ratios each of which is usually constant.

Group (*a*). In systems with a constant ratio the variations of *Rc* hardly ever rpass the limits set by the accuracy of the method, namely, about $\pm 8\%$. Examples

of this group are the opener of *Cambarus* and *Panulirus*, the stretcher of *Panulirus* and several other systems, including the slow bender of *Pachygrapsus*, which is the most easily inhibited of any of the systems studied.

Group (*b*). Variable ratios are found most commonly in preparations which show a high ratio. It is likely that here the factors of facilitation and fatigue of the contraction and of the inhibition play a large part, which explains the difference in values often obtained in one preparation. It is possible, nevertheless, that there are here also real and rather large differences in the effectiveness of inhibition in different preparations. Though in this group the limits are much wider than in those in which the ratio is constant, it is quite possible to determine a mean value which in most cases does not differ from the extremes by more than ±15%. Examples of this group are the closer inhibitor-fast closer systems of all the animals in which inhibition of this system is possible. In Table 5 the mean values alone are given for such variable systems and are marked with an asterisk.

Table 4. *Constancy of more than one ratio in the same system*

Animal	Inhibitor	Excitor	*Fi*	*Fe*	*Rc*	*Fi*	*Fe*	*Rc*
Loxorhynchus	I	Opener	13	30	0·43	17	30	0·57
			20	45	0·45	25	45	0·55
			27	60	0·45	34	60	0·57
			44	100	0·44	53	100	0·53
Loxorhynchus	II	Stretcher	12	30	0·40	14	20	0·70
			20	45	0·44	22	30	0·73
			27	60	0·45	33	45	0·73
			47	100	0·47	44	60	0·73
Panulirus	III	Slow extensor	26	45	0·58			
			35	60	0·58			
			63	100	0·63			
			11	25	0·44			
			13	30	0·43			
			22	45	0·49			
			26	60	0·44			
			47	100	0·47			

Group (*c*). The true opener inhibitors of the crabs are examples of systems which show two distinct values for *Rc*, each of which is remarkably constant. In this group one preparation may jump from one value to the other in two successive determinations, though most frequently the ratio in one preparation is constant at one value while that of another preparation (which may be of another leg of the same animal) is constant at the other. In Table 4 the values have been given for *Loxorhynchus* and for the slow extensor of *Panulirus*; in the two muscles of the crab the values are from different preparations, in the lobster the values were found in the same preparation.

The results of the determinations of *Rc* are given in Table 5 and Table 6. In Table 5 the ratios for the opener, closer, stretcher, and bender of all the animals are given, while Table 6 presents the results obtained from the extensor, flexor and accessory flexor in addition to those from the four distal muscles of the lobster

Effectiveness of inhibition in the opener muscle

The opener inhibitor of *Cambarus* has been previously reported to have a very ective inhibitory effect, giving an *Rc* of 0·41 (Marmont & Wiersma, 1938). In present investigation in which more preparations have been used our mean ue was also 0·41.

The opener inhibitor of *Panulirus* gives a ratio of 0·50, which indicates that ctly two excitatory impulses can be suppressed by a single inhibitory one.

The true opener inhibitor of the crabs shows, as has been pointed out, two tinct ratios. Both these ratios are about the same in the two species of *Cancer*, are definitely higher than the corresponding ones in *Pachygrapsus* and *xorhynchus*, the lower ones in the *Cancers* being of the same order as the higher es in the two other species.

The ratio for the common inhibitor in the crabs is always definitely higher than of the ratios of the true opener inhibitor, and the values are in general much re variable. The ratios in the two species of *Cancer* for this inhibitor are, in trast with the ones of the true opener inhibitor, lower than those of the other genera.

Table 5. *Rc values for the inhibitory systems of the four most distal muscles*

Animal	Opener		Stretcher		Fast closer	Slow closer	Fast bender	Slow bender
	I	III	II	III	III	III	III	III
Panulirus	0·50	—	0·5*	—	1·25*	0·80	1·25	0·75
Cambarus	0·41	—	0·41	—	∞	5·*	1·25*	0·70
clarkii			0·65					
Cancer	0·60	1·0*	0·5	1·0*	1·0*	0·75*	1·00	0·75
anthonyi	0·75		0·6					0·55*
Cancer	0·53	0·93	0·53	1·0*	1·3*	0·65*	1·1*	0·50*
antennarius	0·75		0·75			0·95*		
Loxorhynchus	0·45	1·5*	0·45*	1·5*	1·5*	1·0*	1·4*	0·58
grandis	0·56		0·75					
Pachygrapsus	0·40	1·40*	0·45*	1·25	∞	0·45*	∞	0·33
crassipes	0·50		0·65			0·6		

* Indicates that these systems show a rather wide variation.

Effectiveness of inhibition in the stretcher muscle

The ratio of the stretcher inhibitor system is in most cases very similar to that the opener inhibitor system. In the majority of the preparations in all animals actly the same ratio was found for the two systems when both inhibitors were mulated simultaneously on the same electrodes.

Sometimes exceptions are encountered in certain preparations, although it is ver found that a constant difference occurs in all preparations of a certain system. some systems it is the opener, in others the stretcher, which is occasionally more fficult to inhibit.

In *Cambarus* the stretcher shows besides the same value as the opener a second lue which is higher. In *Panulirus* the ratio is less constant than that of the ener, but does not show a definite second ratio.

The true stretcher inhibitor of the crabs shows two ratios which are not always exactly the same as those of the opener. Both lower and higher values are found as can be seen from Table 5. In *Loxorhynchus* this is clearly demonstrated in Table 4, in which the lower values for the two muscles are about the same, but the higher values are different, that of the stretcher being noticeably higher.

The common inhibitor-stretcher systems of the different crabs give essentially the same ratios as those of the common inhibitor-opener systems of the same species.

Effectiveness of inhibition in the closer muscle

In all of the animals the slow closer contraction can be inhibited, and in most of them with relative ease. The most outstanding exception is the slow closer contraction of *Cambarus*, which, as was pointed out by Marmont & Wiersma (1938) can be inhibited only at low frequencies of excitation and at these only with difficulty. The very high *Rc* which they reported (6·2) has been confirmed by our observations, which gave ratios of the same order, approximately 5·0. The other slow closer systems show a range from about 1·0 in *Loxorhynchus* down to values as low as 0·4 in *Pachygrapsus*.

The fast closer contraction is not inhibitable in either *Cambarus* or *Pachygrapsus*. In both of these cases the muscle responds with a single twitch to a single impulse in the fast fibre, although this contraction in *Pachygrapsus* is relatively weak and in *Cambarus* is very strong. Not only was it impossible to inhibit the twitch contractions, but inhibition also was found to have no effect whatsoever on tetanic contractions. In the other animals where no visible contraction is obtained on a single impulse, there is definite inhibition although the ratios are either about 1·0 or well above it.

Effectiveness of inhibition in the bender muscle

As in the closers there are always two types of contraction in the bender. Again, the slow contraction is in every case easier to inhibit than the fast. In general the values for the fast contractions are about the same as those for the fast contractions of the closer muscle in the same species, but *Cambarus* presents a most interesting exception. In this animal both the slow and fast bender contractions are easily inhibited, the slow showing a ratio of about 0·7, the fast one of 1·2, values which check closely with those obtained by Marmont & Wiersma (1938) even though the latter determinations were made on a much smaller number of preparations. In *Pachygrapsus*, however, the fast bender is, as the closer, uninhibitable. In this animal, in contrast to *Cambarus*, a single impulse in the fibre for the fast bender gives a rather strong twitch contraction of the muscle. The fact that the slow bender contraction of *Pachygrapsus* shows a very low and constant value of 0·3, the lowest ratio obtained in any preparation, has already been mentioned. In general the slow bender contractions appear to show a definitely lower ratio than the corresponding slow closer contractions.

The effectiveness of inhibition in the muscles of the meropodite of Panulirus

As has been mentioned, in Table 6 are summarized the results from the muscles the meropodite in the lobster. There are several points on which some comment ould be made. The fast extensor contraction is here rather easily inhibited and lds the lowest value found for any fast contraction, namely, 0·80. It is interesting note that this value for the *fast* extensor is much lower than that of the other fast tems innervated by the same inhibitor (closer and bender), and corresponds ite well with the values obtained for the similarly innervated *slow* bender and *w* closer.

The four contractions of the main flexor show clearly that the 'slower' the system the more easily it is inhibited. This enlarges the findings of van Harreveld & iersma in this muscle (1939). It may be noted that only the *slowest* flexor ntraction is inhibited with about the same effectiveness as the opener which is hibited by the same fibre. The fastest of the main flexor contractions is very fficult to inhibit, and the values tend to be quite inconstant, ranging from out 1·5 to 3·0.

Table 6. *Rc values for the inhibitory systems of* Panulirus interruptus

System	Opener I	Stretcher II	Closer III		Bender III	
			Fast	Slow	Fast	Slow
Rc	0·50	0·5*	1·25*	0·80	1·25	0·75

System	Extensor III		Accessory Flexor III	Flexor I			
	Fast	Slow		Fast	2nd fast	2nd slow	Slow
Rc	0·8	0·45 0·6	0·60	2·0*	0·80	0·70	0·52

* Indicates that these systems show a rather wide variation.

C. The presence or absence of supplementary inhibition

Marmont & Wiersma (1938) have shown that in the opener of *Cambarus* duction of the muscle action potentials during inhibition is present only when ie inhibitory impulses arrive within a restricted time limit before the excitatory nes. Inhibition in which the muscle action potentials are reduced has been called upplementary as against simple in which no reduction occurs. Wiersma & Helfer 941) have shown that in certain inhibitor systems of *Cancer* no supplementary hibition can be obtained no matter at what interval the excitatory and inhibitory npulses arrived at the muscle. These systems, therefore, show simple inhibition nly. This was shown to be the case in the inhibition of the slow closer and of the pener by the common inhibitor. The true opener inhibitor of *Cancer* did, however, now supplementary inhibition which is in accord with the findings of Marmont Wiersma (1938) on several other crabs. The present investigation concerns itself ainly with the determination of which systems are capable of giving supple-entary inhibition and which ones are not. In contrast with the earlier papers ie quantitative effects were treated more superficially.

In *Cambarus* it was found that besides the opener, the stretcher can be made to show reduction in muscle action potentials during inhibition. Marmont & Wiersma (1938) described the phenomenon for the opener of the crayfish thus: 'the action currents are reduced somewhat gradually at the onset of the supplemented inhibition but rise immediately (rebound) to their normal height when the inhibiting impulses are stopped.' In the present investigation the gradual onset and sudden rebound was also observed for the stretcher system (Fig. 2 A). No sign of reduction of the action potentials was found in the inhibition of the bender and closer contractions in the crayfish. It is of particular interest to note that the inhibitor which produces the phenomenon in the stretcher fails to do so in the closer.

It was found possible to elicit supplementary inhibition in both the true opener inhibitor and the true stretcher inhibitor system in all the crabs (Fig. 2 B). In all cases the supplementary inhibition shows the characteristics reported by Wiersma & Helfer (1941) for the opener system of *Cancer*, namely, the gradual depression at the onset of inhibition and the gradual growth upon release in contrast to the

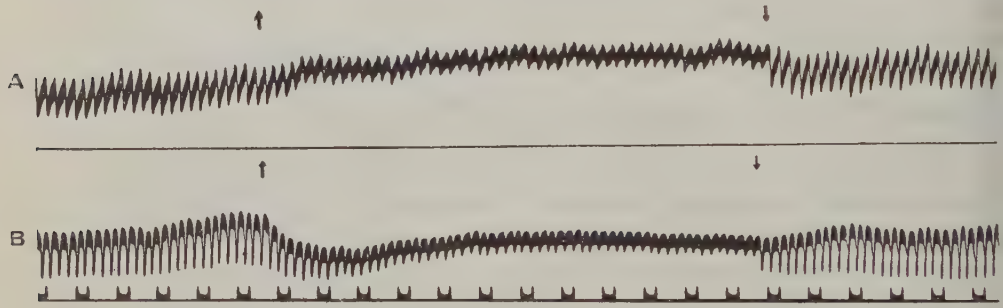

Fig. 2. Supplementary inhibition of action potentials of the stretcher muscle. A. *Cambarus*. Notice the immediate rebound to full height at the end of inhibition. B, *Cancer anthonyi*. At the end of inhibition the action potentials grow gradually to full height. ↑ = onset of inhibition. ↓ = release of inhibition. In both cases the inhibitory stimulus was given 2 msec. before the excitatory one. Frequency 45 per sec. Time 0·1 sec.

immediate rebound of the crayfish. In no case could supplementary inhibition be obtained with the common inhibitor. This enlarges the findings of Wiersma & Helfer (1941) with this inhibitor for the opener and the closer to include the other muscles which it innervates. Especial attention has been paid to the inhibition of the slow bender contraction of *Pachygrapsus* because it is the most easily inhibited contraction. Nevertheless, we have never obtained any signs of supplementary inhibition in this muscle. It should be pointed out in this regard that the slow closer and the slow bender contractions of *Pachygrapsus* do not lend themselves very well to this sort of investigation because of the small magnitude of the muscle-action potentials which they give (in sharp contrast to the very large magnitude of the fast-action potentials of these muscles). We have not paid especial attention to the presence or absence of the small depression of facilitation and the subsequent growth of the muscle-action potentials on release of simple inhibition which Wiersma & Helfer (1941) reported, but noted that it was present in several cases.

In *Panulirus* not one of the systems has ever shown any signs of supplementary hibition. The four most distal muscles and the extensor have been carefully amined in this respect. As the complete absence of action-potential reduction at least the opener and stretcher muscles was unexpected, a large number of eparations of the opener and of the stretcher have been made, but not one of se has given any indication of the phenomenon.

DISCUSSION

It is surprising that the ratio of the frequency of inhibitory impulses to the citatory impulses for just complete inhibition is constant with changes in the equencies of stimulation. Such constancy has been seen in nearly all of the eparations observed. The question arises how this constancy of *Rc* can be brought out. It is a well-established fact that in the muscles of decapod Crustacea the ength of a contraction depends on the excitatory frequency, and that with crease in frequency there is in these muscles not only an increase in contraction ength by algebraic summation but also by facilitation,[1] which results in successive rve impulses becoming more and more effective. The effect increases the quicker e impulses follow each other. That this facilitation does not make contractions tained on higher frequency stimulation relatively more difficult to inhibit than low frequencies, which would result in an increase in *Rc*, can find its explanation ly in a simultaneous increase in effectiveness of the inhibitory processes. It can proven that this is indeed the case. If inhibition is started before excitation it is und that it can completely suppress a contraction caused by a frequency of citation which it cannot suppress if both are started at the same time. Wiersma van Harreveld (1934) found in *Eupagurus bernhardus* that if a frequency of hibition is selected which cannot suppress but can only slightly reduce a test ntraction when both are started at the same time, continued stimulation of the hibitor suppresses successive test contractions more and more until fatigue of e inhibitory mechanism sets in and the contractions become again less completely ppressed. Under the same circumstances all inhibitory systems were observed give similar results. The counterpart of this experiment, i.e. to test the influence short inhibitions during continuous stimulation of the excitor, was performed th the following result: stimulating at frequencies near *Rc* values the effect of e inhibitions becomes gradually less and less, even during the time in which the ntraction begins to diminish through fatigue. This shows that facilitation of the ntraction makes it less inhibitable, even if the mechanical effect diminishes. can thus be concluded that both excitation and inhibition undergo facilitation d that these facilitations balance each other at the *Rc* values.

Although the *Rc* value gives a measure of the relative rate of the facilitations of

[1] Facilitation of excitation has been shown to work at two places, for there is a facilitation of e action potential and of the contractile mechanism (Wiersma & van Harreveld, 1939). In the esent discussion we have taken these two together, thus deliberately simplifying the picture. It ems possible that in the inhibitory process two similar facilitations are active, but at present it is t fruitful to discuss the theoretical consequences of such an arrangement.

excitation and inhibition of a system it is not possible to measure the absolute rates. However, the results obtained point to some conclusions. In a doubly motor innervated muscle inhibited by one fibre it is likely, for instance, that the two ratios for the two contractions can be taken as a measure of the relative strength of the excitatory facilitations. It thus becomes evident that in such cases as the fast closer contraction of *Cambarus* and the fast closer and fast bender contractions of *Pachygrapsus*, which are uninhibitable at any frequency, the ineffectiveness of inhibition seems to be directly associated with the excitatory process. In the case of the opener and stretcher, which usually show the same ratio, it is logical to conclude that the two facilitations, inhibitory and excitatory, of these muscles are the same. In the cases in which there is a difference in the ratios of these two muscles this will be most probably due to a difference in the inhibitory facilitations since it seems unlikely that the excitatory facilitation of the same motor axon would change in one muscle and not in the other.

Since they share a single inhibitory fibre it might appear logical to consider the similarity of the *Rc* values for the fast extensor, the slow bender, and the slow closer systems of *Panulirus* as indicative of a similarity in the facilitation of the excitatory processes of these contractions. That such is not necessarily the case, however, is seen in *Cambarus*, in which the closer and stretcher are innervated by the same inhibitor, yet the stretcher contraction gives *Rc* values which are of the order of one-tenth those of the slow closer contraction. Any tenfold difference between the facilitation of these contractions is certainly not present.

It is clear that constant ratios depend on a great number of factors. If there is still facilitation of excitation or of inhibition from a preceding volley the ratio will shift to one side or the other. This will show at the same frequency on repetition. This factor can be excluded, however, by allowing sufficient time between stimulations and also by very short durations of the tests. Both have been utilized as much as practicable in the experiments. That the fast systems have, nevertheless, given rather inconstant ratios may well be due to the fact that these systems are generally rather inconstant. For instance, repetition of the same stimulation often results in contractions which are noticeably different, even though a prolonged rest period is given, which may well indicate variations in the facilitation of the excitation.

The appearance in certain preparations of two often well-defined ratios cannot be considered to be due to such an inconstancy. This type of variation is presumably due to a shift of one or the other of the facilitations to a different level which is the same in different preparations. This shift must be a very sudden one in those instances in which the second level was observed shortly after the first in the same preparation. The reasons for such a shift and for the constancy of the two levels are unknown.

The incomplete inhibitability of certain preparations at the higher frequencies of excitation which are completely inhibitable at lower frequencies is, however, presumably not due to any peculiarity of the facilitations. The most likely explanation is that the inhibitory mechanism is stimulated with too high a frequency

l that certain of the impulses fail to have an effect. That this explanation is sonable is shown by the fact that in preparations in which the ratio is favourable he inhibitor, the excitor may fail first in a similar way. In such a case the ratio be constant up to high values of excitation but will drop at still higher ones. nay be remarked that failure of excitation or inhibition does not influence the o of subsequent contractions at lower frequency levels, these being quite normal.

In crabs the opener and stretcher muscles are inhibited by two different fibres. e functional significance of this arrangement is unknown (see Wiersma, 1941), it is of interest to note that the *Rc* values of the two inhibitors show a certain tion. In the two *Cancers*, where the true opener inhibitor is relatively ineffective, common one is relatively effective, whereas in *Pachygrapsus* and *Loxorhynchus* elatively effective true opener inhibitor is accompanied by a rather ineffective nmon inhibitor.

In previous papers it has been pointed out that the facilitation of the excitatory chanism is most likely not due to spatial facilitation by the involvement of more more muscle fibres but to a gradual increase of contraction strength in each scle fibre. This means that the muscle fibre of the crustaceans does not contract an all-or-none fashion. The results obtained with inhibition give additional port to this view. The absence of any effect on the muscle action potentials in st inhibitions shows that the inhibitory mechanism must be located after the cess of this muscle action potential and since there is an innervation of each scle fibre in many places the inhibition must be present at all these places. is thus thought that inhibition like excitation is a gradual process in each muscle re, or better, at each nerve ending on each muscle fibre.

One of the most significant observations arising from the present investigation the apparent lack of correlation between the ease with which an inhibitor is able suppress the contraction of the muscle which it innervates and the presence of e phenomenon of supplementary inhibition. It would seem quite plausible that obtain a maximum efficiency of the inhibitory process it would be necessary t only to block the transmission step between the muscle action potential and e contractile mechanism, but to suppress the earlier transmission step between e nerve action potential and the muscle action potential as well. This is, however, finitely not the means whereby certain of those systems showing a low *Rc* achieve eir effectiveness, e.g. the opener system of *Panulirus* and the slow bender stem of *Pachygrapsus*.

The presence of supplementary inhibition in both the opener and stretcher uscles of crayfish and crabs, muscles which have separate inhibitory axons, shows at the phenomenon is not limited to one inhibitor. At the same time the absence the phenomenon in other muscles inhibited by these same inhibitors, for instance e closer of *Cambarus*, indicates clearly that the mechanism involved in supplementary inhibition is not to be found in the inhibitory innervation, but in the uscle itself. It is apparently an extra process whereby inhibition can be assured those few systems in which the phenomenon can be demonstrated. *Panulirus* ems to be totally without this type of inhibition.

SUMMARY

The effectiveness with which different contractions in a number of muscles can be inhibited was investigated. As a measure of this effectiveness the frequency of inhibition which can just inhibit a contraction with a given frequency of excitation was determined. It was found that in all systems the ratio (*Rc*) of such inhibitory frequencies to that of the excitatory frequencies they can suppress was constant for a wide range of frequencies.

At high frequencies either the inhibition or the excitation may become less effective. This is explained by failure of the respective system to function normally at such a frequency.

The effectiveness of inhibition of different systems was determined. Some systems show a very constant *Rc* value; in a second group *Rc* varies within wider limits; and a third group shows two distinct *Rc*'s sometimes in the same preparation at different times.

Rc values have been found to vary widely. For instance, in the bender inhibitor–slow bender system of *Pachygrapsus* three excitatory impulses are suppressed by one inhibitory impulse; in the closer inhibitor-slow closer system of *Cambarus* one excitatory impulse needs five inhibitory impulses to counteract its effect. The fast closer contraction of *Cambarus* and the fast closer and fast bender contraction of *Pachygrapsus* were found to be uninhibitable, i.e. no effect of inhibition whatsoever was noticed on any of these contractions. All three systems are distinguished by giving a mechanical response to a single stimulus in contrast with all the inhibitable systems which do not respond to single impulses.

Reduction of the action potentials during inhibition is obtainable in only a few systems, namely, the opener inhibitor-opener and the stretcher inhibitor-stretcher systems of *Cambarus* and the crabs. (In the crabs this applies only to the 'true' inhibitors.) In all other systems, including every system of *Panulirus*, no reduction of the muscle action potential is obtained.

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CARBOHYDRATE METABOLISM OF CHICK FIBROBLASTS IN VITRO

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(With Nine Text-figures)

INTRODUCTION

ER since Warburg and his school (1930) called attention to the quantity of lactic d produced by cancerous tissue, the carbohydrate metabolism of growing cells s been a matter of interest. The provision of energy for the synthetic processes growth and for the actual process of cell division has been much discussed, and hough the latter has been shown to be insignificant in amount, the former is less viously so. More recent work makes it extremely doubtful if there is really any ndamental connexion between growth and glycolysis. Boyland & Boyland (1939) owed that there was practically no correlation between the rate of growth of nours and their glycolytic powers. This applied to sarcomata and carcinomata ke. Berenblum *et al.* (1940) have also shown that the carbohydrate metabolisms normal and carcinomatous skin are almost identical. From these experiments en it seems fairly clear that lactic acid formation and growth are, at the most, lly indirectly linked. On the other hand, the carbohydrate metabolism of embryos, many ways resembling that of malignant tissues, appears to have certain culiarities when compared with that of the adult animal, and there is evidence at the breakdown of sugar follows a path in which the phosphorylation of the gar appears to be a less important factor, if not completely unnecessary. Pomerat Willmer (1939) showed that substances which inhibit the phosphorylation echanism did not necessarily inhibit the growth of fibroblasts in culture, but that nen the direct glucose breakdown was interfered with, then growth suffered also. ne experiments were not, however, conclusive, since the inhibitors used were not ecific and other essential processes may well have been adversely affected.

The growth-promoting power of an extract of an adult tissue in Tyrode solution in general, proportional to the glycolytic power of the tissue from which it is rived; and, finally, there is the undoubted fact that many rapidly growing tissues produce considerable quantities of lactic acid.

Taking all these things into consideration, therefore, it seemed worth while study the carbohydrate metabolism of fibroblast cultures during a period of iescence in a plasma and Tyrode medium and to observe the effects of adding

an extract of embryonic tissues which is known to have certain well-defined accelerating effects upon the growth of the tissue as well as upon the migratory activity of the cells, a process which is closely linked with their growth. This study could be coupled with observations upon the carbohydrate metabolism of colonies growing continuously under the influence of embryonic juice and of those in a state of prolonged survival in a Tyrode-plasma medium.

Previous work had shown that the addition of embryo juice to a quiescent culture of either heart or bone fibroblasts immediately causes the cells to become more active, and after about 12 hr. the cells start to divide. It should be enlightening therefore to see if this addition of embryo juice produced any significant and coincident change in the carbohydrate metabolism, as measured by the glucose consumption. These considerations led up to the experiments described in the present paper which later came to cover a much wider field. The reason for this was that owing to the complexity of the problem involved and the difficulties in dealing with changes on such a small scale, new techniques had to be evolved, and in order to amplify and interpret the data obtained, measurements of the lactic acid production were made at the same time as the glucose estimations.

EXPERIMENTAL RESULTS

(1) The effects of the addition of embryo juice on the glucose consumption of surviving cultures

The amount of sugar utilized by a tissue is obviously dependent upon the amount of tissue present, and in the case of tissue cultures it is difficult to obtain exact data of this sort and impossible to determine the amount of tissue present at two different times during an experiment, since all the available methods involve the death of the tissue. On the other hand, by measuring the glucose consumption continuously before and after the addition of embryo juice, and knowing that under such conditions cell divisions do not occur till some 12 hr. after the addition of the juice, comparative figures can be obtained and the actual mass of the tissue becomes of less importance, although even then it should be remembered that the increase in cell mass generally precedes cell division.

For the actual experiments ten cultures of osteoblasts from the os frontale of the 12-day chick embryo, generally after their fourth transplantation in vitro, were transferred to Carrel flasks. The solid phase of the medium used was composed of 0·2 c.c. of adult fowl plasma and 0·2 c.c. of Tyrode solution. After clotting of this medium had occurred 0·5 c.c. of Tyrode solution was added as a supernatant fluid. Time was allowed for equilibration of the glucose between fluid, coagulum and tissue, and the flasks were well agitated before 0·05 c.c. samples of the supernatant fluid were taken for analysis. The sugar was estimated by the method for 'true sugar' described by Haslewood & Strookman (1939), as this was found to be reasonably satisfactory and has the advantage that many estimations can be carried out in a short time. It was found to be better than the cerium sulphate methods for the small quantities involved. The original sugar in the medium was found to

a variable quantity since the plasma content was variable, generally being mething of the order of 200 mg. %, a figure very much higher than that of mmalian plasma. In these first experiments the Tyrode solution was made up h 100 mg. %.

A large number of flasks were set up for these experiments, and the results those in which all the cultures remained healthy and active throughout the eriment are shown in Fig. 1. It will be observed that the utilization or dis-pearance of sugar during the initial phase of 48 hr. is very variable. The mass tissue in each flask was seldom the same, since, although in setting up the flasks cultures were always used, there was no method of controlling their mass except ghly by visual observation. The cultures had to be small so that questions of gen penetration should not arise, and so that there should be no question of olysis occurring in the centre of the implant and thus producing substances

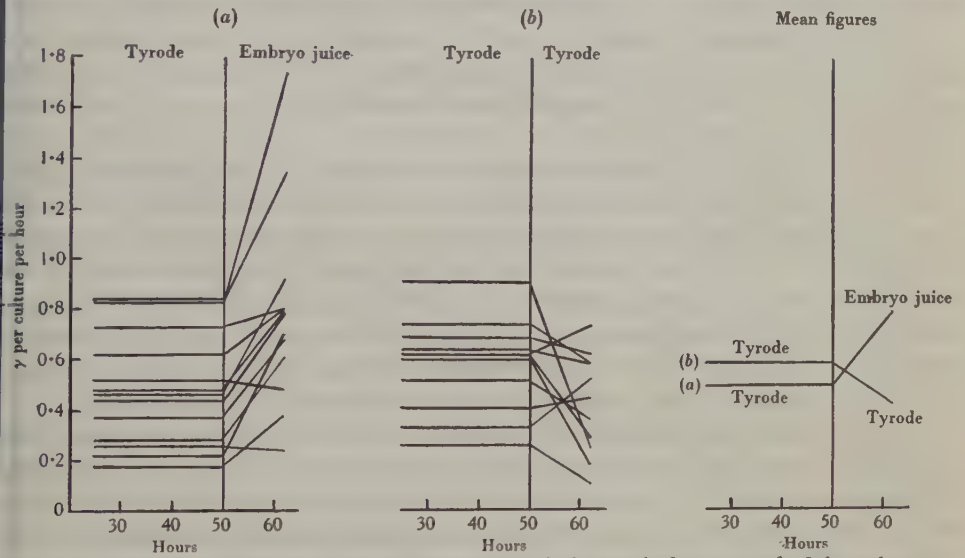


Fig. 1. Mean sugar utilization per culture per hour before and after renewal of the culture medium with (*a*) embryo juice (30 %) and (*b*) Tyrode.

hich might have altered the metabolism of the rest of the cells. On the other nd, the tissues could not be very small, since it was necessary for them to be ble to cause measurable changes in the sugar concentration.

At the end of 48–50 hr. the original fluid medium was removed and either newed with 0·5 c.c. of fresh Tyrode or with 0·5 c.c. of embryo juice (30 % in yrode). The new medium was allowed to equilibrate for an hour or so, and then e glucose present was estimated. The cultures were then incubated again and adings taken for the next 24 hr. It is clear from the figure that, on the whole, mbryo juice tended to raise the glucose consumption, whereas the addition of yrode solution tended to have the opposite effect. Moreover, the higher the itial rate of glucose disappearance the greater tended to be the effects. Although e results of these experiments were not as uniform as one could wish, it seemed

to be clear that embryo juice hastened the removal of sugar from the medium by these cultures, whilst simply renewing the Tyrode had the opposite effect. There was plenty of sugar available in both media throughout the experiment.

At this point it is necessary to inquire what happens to the sugar which disappears, and to attempt some correlation with the visible effects of the Tyrode and embryo juice on the behaviour of the cultures. The addition of embryo juice to cultures after 48 hr. in Tyrode immediately causes increased protoplasmic activity, cell movement, and the formation of intracellular fat droplets. Cell divisions begin to occur after about 12 hr. None of these changes follow the addition of Tyrode, after which the cells remain quiescent and healthy with clear hyaline cytoplasm throughout the experiment. The disappearance of the glucose from the medium must mean either direct utilization by oxidation or conversion to lactic acid, or it must be stored either as glycogen or fat. Glycogen is not detectable by histological methods in such cultures, suggesting that probably the sugar is directly utilized, though the appearance of the fat has still to be accounted for. Experiments to be described later make this explanation probable, and the extra sugar utilization would parallel the extra movements of the cells with which it is coincident in time. Cell divisions occur later.

A series of experiments on the glucose consumption of fourth passage osteoblast cultures planted directly into flasks with either embryo juice or Tyrode as the fluid phase of the medium showed a marked difference in behaviour in the two media. In embryo juice the glucose consumption was very high initially and rapidly fell off. The figures obtained agreed substantially with those found by Gey *et al.* (1940) for sarcoma cells under similar conditions and with the earlier figures obtained by Krontowski & Jazimirska-Krontowska (1926) on hanging-drop cultures of fibroblasts. In Tyrode solution the utilization was on the whole much steadier.

The glucose utilization appeared to be but little affected by the actual concentration of sugar in the medium (initially either about 100 or 200 mg. %), except that the uptake may have been accelerated in the higher concentration during the first day but slowed down later, so that after 3 days the total utilization in both media was much the same.

These experiments gave incomplete information in that the actual weight of tissue present was unknown, so that it was impossible to express the data in terms of the mass of tissue present. However, they clearly showed a difference in the time course of the sugar utilization between cultures fed with embryo juice and those in Tyrode. Embryo juice determined a rapid initial uptake of glucose.

Carbohydrate metabolism and nucleoprotein phosphorus content

In order to obtain more reliable data on the metabolism of the tissues it was decided to adapt the method of Berenblum *et al.* (1939) for the micro-determination of nucleic acid phosphorus for the measurement of the growth of tissue cultures. The actual measurement of growth is extremely difficult owing to lack of a suitable

erion. Total weight of tissue cultures is unsatisfactory, since the cultures can y with difficulty be separated from the plasma coagulum, though there are thods which can be used for this purpose. But even if the culture is satisfactorily arated from the medium the actual amount of protoplasm present is uncertain ng to imbibition of water by the cells, accumulation of fat or even the develop- nt of intercellular substances. For example, Laser (1933 *a*) found that cultures wn in serum weighed more than corresponding cultures in embryo juice even ugh cell divisions are far more numerous in the latter. The total number of cells sent, together with some estimate of their size, is really what is required. This be estimated in the periphery of a culture by photographic means but this is use when the metabolism of the culture as a whole is required. Every cell has ucleus and each nucleus appears to 'control' a certain amount of cytoplasm ll therefore an estimate of the amount of nuclear material would give the data uired. The most characteristic constituent of the leus is nucleoprotein, and with one or two exceptions the nucleoprotein present in a tissue is present in nucleus. Consequently estimations of nucleoprotein sphorus can be used for estimating the amount of lear material, hence of nuclei and hence of total ue present. There are two possible sources of error in method: first, in some tissues nucleoprotein is present he cytoplasm and this probably has no bearing on the ount of nuclear material; secondly, the method em- yed estimates the amount of phosphorus which is not ractable in alcohol and chloroform at 65° C. followed *N*/10 hydrochloric acid. Phosphoproteins may be in- ded in the estimates. Before it can be used as a method measuring growth the behaviour of the particular tissue s first to be studied, since it has been found that the cleoprotein content of all tissues does not behave in e same way under the same treatment. The method s the advantage that the plasma coagulum in which the tissues are embedded can main during the estimations and gives only a small and reasonably constant blank. r some obscure reason the blank increases very slightly after the coagulum has en incubated for a few days, especially in the presence of embryo juice.

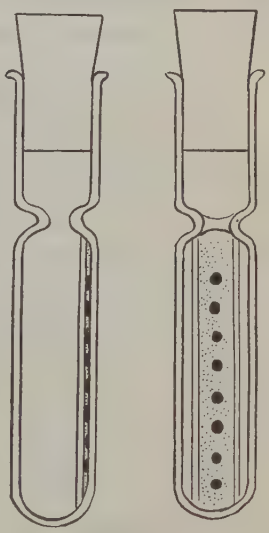


Fig. 2.

The method adopted has been to grow eight cultures in a modified roller tube, the minimum of plasma (0·2 c.c.) and 0·5 c.c. of supernatant fluid. The tubes ere made from monax glass test-tubes by putting a constriction in them at about cm. from the bottom and cutting them off about 2·5 cm. above the constriction. he tube and the arrangement of the cultures are shown in Fig. 2. The tubes were osed during incubation by rubber stoppers and were placed on a circular rack d rotated slowly and constantly by means of a Synclox motor during the time incubation at 38° C. Fluid can be drawn off at any time for estimation, and hen the amount of nucleoprotein present has to be determined the whole extraction

prior to the estimation can be carried out in the same tube, thus avoiding any loss, and at the same time simplifying and standardizing the extraction.

In these experiments with modified roller tubes the sugar was again estimated by the method of Haslewood & Strookman, 0·05 c.c. samples being taken. The lactic acid was measured on 0·4 c.c. samples by the method of Friedemann *et al.* (1927), which although hardly sensitive enough for the amounts of lactate present in some of the samples, was found to give reproducible results, and considered to be superior to manometric methods owing to its greater specificity.

It was first necessary to work out the results on a series of blanks, that is to say on tubes containing medium but no tissue, in order to find out the amounts of nucleoprotein phosphorus present, and to determine whether any glucose consumption or lactic acid production occurs in the medium. The accompanying Table 1 gives the results of these blank determinations. It is clear that in both Tyrode and embryonic juice there appears to be a slight increase in the nucleoprotein phosphorus. There is no glycolysis in the plasma-Tyrode medium, but the

Table 1. *The changes which occur in the medium during* 4 *days at* 38° C.

Medium	Initial			After 4 days		
	N.P.P. γ	Glucose mg.	Lactic acid mg.	N.P.P. γ	Glucose mg.	Lactic acid mg.
Plasma 0·2 c.c. Tyrode 0·5 c.c.	0·26 ± 0·03 (10)	1·45 ± 0·05 (5)	0·080 ± 0·008 (6)	0·30 ± 0·02 (15)	1·42 ± 0·05 (8)	0·073 ± 0·007 (7)
Plasma 0·2 c.c. *15 % embryo juice 0·5 c.c.	0·24 ± 0·03 (7)	1·41 ± 0·05 (6)	0·09 ± 0·01 (7)	0·33 ± 0·03 (10)	1·31 ± 0·02 (10)	0·11 ± 0·01 (8)

N.P.P. = nucleoprotein phosphorus.

The numbers in brackets indicate the number of experiments upon which the average values are based.

* In some later experiments 30 % embryo juice was used with similar results.

figures suggest that perhaps a little may occur in the embryo juice medium. In the figures given for the changes occurring when tissues are present account has been taken of those caused by the medium alone.

The first series of experiments was carried out upon chick osteoblasts (and/or periosteal fibroblasts). The cells were obtained as before from the frontal bone of the 12-day embryo chick, and they were subcultured in plasma and 15 % embryo juice for at least four passages by the hanging-drop method before being transferred to the roller tubes for experimental purposes. In interpreting the results obtained it is only possible to judge the general trend of events owing to the initial and uncontrollable variations in weight of the tissues used and the consequent differences in amounts of metabolites used or produced. Moreover, the micromethods have their own significant margins of error. For these reasons the actual experimenta

a have all been plotted and curves drawn where they seemed most in accord n the observations. The majority of estimations were made as follows: (1) im- diately after the cultures were put up and equilibrium was established (in ctice this was between 3 and 4 hr. after adding the fluid phase); (2) 48 hr. after first readings; and (3) 48 hr. after the second readings; (4) occasional estimations e made at other times, chiefly at 28 and 76 hr.

The experiments with osteoblasts fall into two groups. In the first, only glucose sumption was considered in relation to nucleoprotein phosphorus, and this measured on cultures growing in 15 % embryo juice. For the estimations of ose consumption several successive readings were made on the sugar content

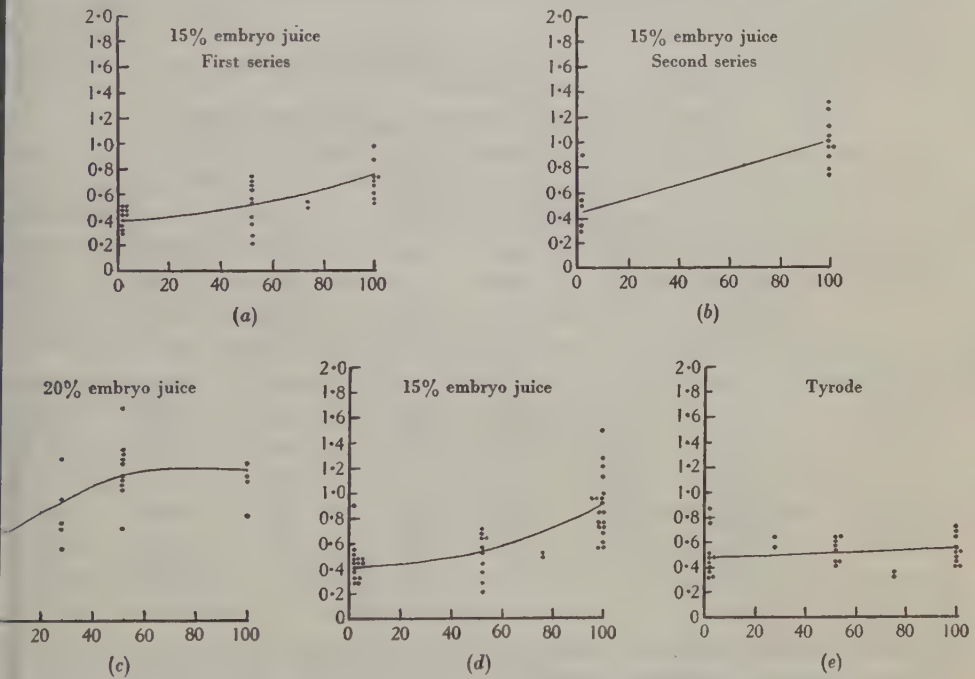


Fig. 3. Nucleoprotein phosphorus content of tissues cultured in different media. Ordinates: nucleoprotein phosphorus (γ). Abscissae: time in hours.

each tube and the results are not strictly comparable with the rest which will be scribed later, but it may be said that they lend very strong support to those found later experiments. These experiments also serve as an introduction to the findings the nucleoprotein content of the cultures which are shown in Fig. 3. It will be en that there is considerable scatter in the values obtained and this scatter creases as the cultures grow. To some extent this is to be expected, for if the ltures double their nucleoprotein in 4 days, as indeed they approximately do, en the difference between the largest and the smallest will also be doubled in at time. It will also be evident that the cultures appear not to gain quite so ickly during the first 2 days as they do in the last 2 days. This is even more

evident when the values for the later experiments in the same medium are added to those of the first series (Fig. 3). It may be remarked here also that in the one series of tubes from which readings were obtained on the sixth day of cultivation there was apparently no rise in nucleoprotein between the fourth and sixth days although the cultures increased in area. The number of experiments was not sufficient to warrant any definite statement, but the results obtained with 20% embryo juice instead of 15% showed a similar phenomenon, but this time the cessation of nucleoprotein increase occurred earlier, at the end of 2 days. The cultures in 20% juice may have started at a slightly higher level, but they doubled their nucleoprotein phosphorus in 2 days and then showed no further increase. Admittedly there are only four points for the fourth day but they belong to two separate experiments from which 2-day readings were also obtained, and the 4-day readings were of the same order as the 2-day readings in both cases. In a medium

Table 2. *Data for osteoblasts, derived from accompanying curves*

Medium	Time in hours	Mean N.P.P. in γ	Glucose utilized in γ	Lactic acid liberated in γ	Glucose N.P.P. per hour	Lactic acid N.P.P. per hour	Lactic acid / Glucose
Tyrode	0–20	0·48	50	80	5·2	8·3	1·6
	20–40	0·50	70	70	7·0	7·0	1·0
	40–60	0·52	120	70	11·5	6·7	0·59
	60–80	0·54	170	70	15·8	6·5	0·41
	80–100	0·56	230	60	20·5	5·4	0·26
15% embryo juice in Tyrode	0–20	0·41	110	(80)	13·5	(9·8)	(0·73)
	20–40	0·44	90	(60)	10·2	(6·8)	(0·67)
	40–60	0·50	80	(40)	8·0	(4·0)	(0·50)
	60–80	0·60	70	(30)	5·9	(2·5)	(0·43)
	80–100	0·78	70	(30)	4·5	(2·0)	(0·44)
20% embryo juice in Tyrode	0–20	0·75	230	150	15·4	10·0	0·65
	20–40	1·0	170	100	8·5	5·0	0·59
	40–60	1·15	130	80	5·7	3·5	0·61
	60–80	1·18	120	60	5·1	2·6	0·59
	80–100	1·14	110	50	4·9	2·2	0·45

of Tyrode solution instead of embryo juice there might perhaps be a slight increase in nucleoprotein phosphorus, but the amount remains practically unchanged during the 4 days of cultivation.

It is clear from these figures, also summarized in Table 2, that, as might be expected from its action in promoting growth and cell division, embryo juice causes an increase in nuclear material, and the rate of increase is proportional to the strength of the embryo juice used. The mitotic index, or the percentage of cells dividing per hour, has previously been shown to be proportional to the concentration of the juice. The extract was not renewed during these experiments, and it is evident that, if the increase in nucleoprotein is really limited in extent as it appears to be, some factor must get exhausted, and a factor required for the building of new tissue. It appears as if the embryo juice accelerates the rate of construction of nucleoprotein, in proportion to its concentration, but that the actual amount of nucleoprotein built is limited by the available supply of some other material.

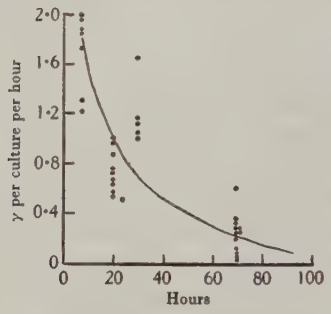
e latter may be in the tissue, the juice or in the plasma; the available data cannot decide which, but they suggest the plasma as the source, since, if 15% juice ermines a two-fold increase and the necessary material were in the juice a 20% ution should determine a 2·7-fold increase, and in that case the 4-day readings 20% juice might be expected to be nearer 1·6 than 1·1, as they actually are. In rode solution alone osteoblast cultures show mitotic figures for the first 48 hr., ugh in ever decreasing numbers, but it is interesting to find that there is no responding measurable increase in the nucleoprotein so that the tissue is pre-nably living on its own reserves with regard to this substance, a point for which re is further confirmation in results to be described later in this paper.

The glucose consumption of the osteoblasts in embryo juice and Tyrode ution may now be considered. In the first series of experiments the glucose ncentration in the unchanged medium was determined from time to time, and amount of sugar which had disappeared, glucose utilization, was recorded. The ncentration of juice was 15%. The results (Fig. 4*a*) were rather irregular for a riety of reasons and the method was subsequently discarded, but as far as they they again confirm the results of Gey *et al.* for the glucose consumption of coma tissue, in that there was at first a very rapid consumption which decreased very small values after 2 or 3 days. The decrease was not due to the diminished pply of available glucose, because, as previously stated, the rate of utilization is rdly affected by the initial concentration between 100 and 200 mg. %.

The results of the second series of experiments on glucose utilization in which e nucleoprotein phosphorus and lactic acid production were determined simul-neously in culture tubes incubated for stated times are shown in Figs. 4 and 5. he corresponding figures for the nucleoprotein are those already shown in Fig. 3. gain there is a wide scatter in the actual values obtained. The absolute amounts glucose found in the medium are plotted. They were calculated from the ncentration and the total volume of medium on the assumption that the sugar as evenly distributed. The changes in the medium without tissue are also plotted. is clear that although the total sugar disappearing in 4 days is not greatly different hether embryo juice is present or not, yet the shape of the curves for the two media e somewhat different. In the presence of embryo juice, as in the earlier experi-ents, the glucose disappearance is most rapid at first and then falls off, while in yrode solution the reverse is the case. From the curves of Fig. 4, Fig. 4*b* has een constructed. This clearly shows the different time course of the glucose sappearance in the two media, and the results are even more striking when the ucose utilization is related to the nucleoprotein phosphorus, that is, to a factor hich is probably nearly proportional to the amount of tissue present. These sults are shown in Fig. 6*a*. The values for the nucleoprotein phosphorus are the ean values for each 20 hr. period.

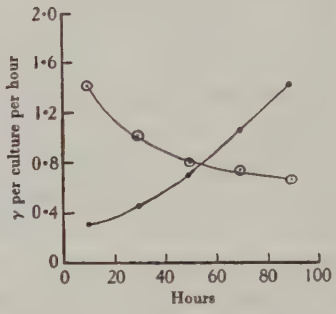
Mutual confirmation of the results is obtained from the corresponding figures r lactic acid production, shown in Figs. 5 and 6*b*, for probably the glucose nsumption and lactic acid production bear some relationship to each other, and is relationship repays examination. The ratio of lactic acid production to glucose

disappearance is plotted in Fig. 6*c*. It is at once obvious that in Tyrode solution the lactic acid produced early in the experiment must come from stored carbohydrate or from some non-carbohydrate source. Glycogen is not generally detectable in cultures of osteoblasts after the first passage or two, and the point made earlier that the tissue in Tyrode and plasma is living on its own resources, together with the hyaline condition of the cytoplasm of the cells, perhaps suggest



(*a*)

Sugar utilization per culture per hour. 15 % embryo juice.



(*b*)

Sugar utilization per culture per hour.
⊙——⊙ 20 % embryo juice.
·——· Tyrode.

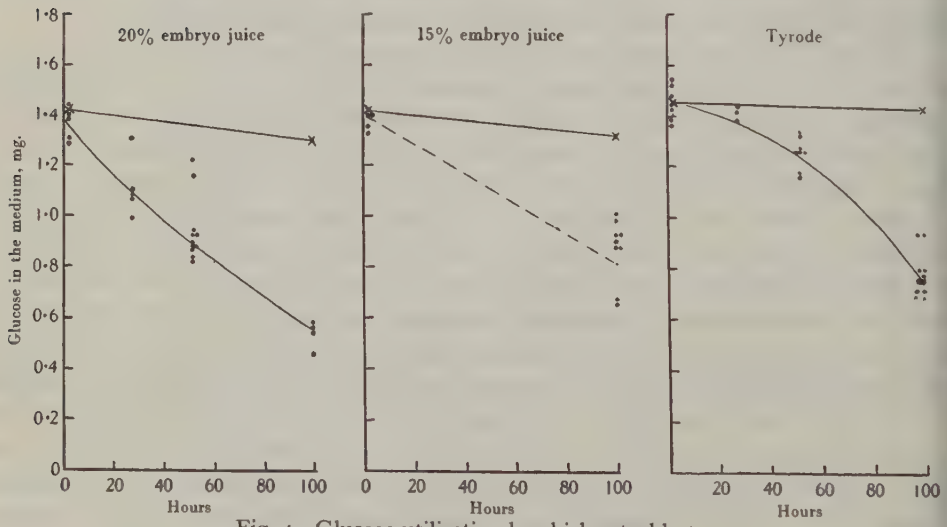
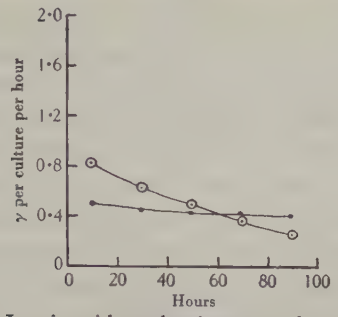


Fig. 4. Glucose utilization by chick osteoblasts.

a fat or protein source. In both media there is a decline in the lactic-glucose ratio, and in both there is a decline in the mitotic rate. These changes are slight in the presence of embryo juice but rapidly proceeding in the Tyrode medium. Perhaps it is possible to correlate high growth rate with high lactic-glucose ratio, assuming also that the embryo juice allows the tissues to pick up glucose more readily from the medium than is possible in Tyrode alone, an assumption which appears to be fully justified by the figures already produced. The lactic-glucose ratio is as a



Lactic acid production per culture per hour.
⊙——⊙ 20 % embryo juice.
•——• Tyrode.

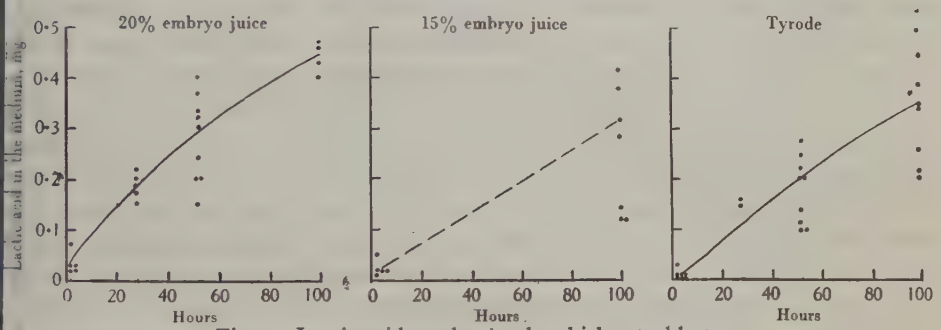


Fig. 5. Lactic acid production by chick osteoblasts.

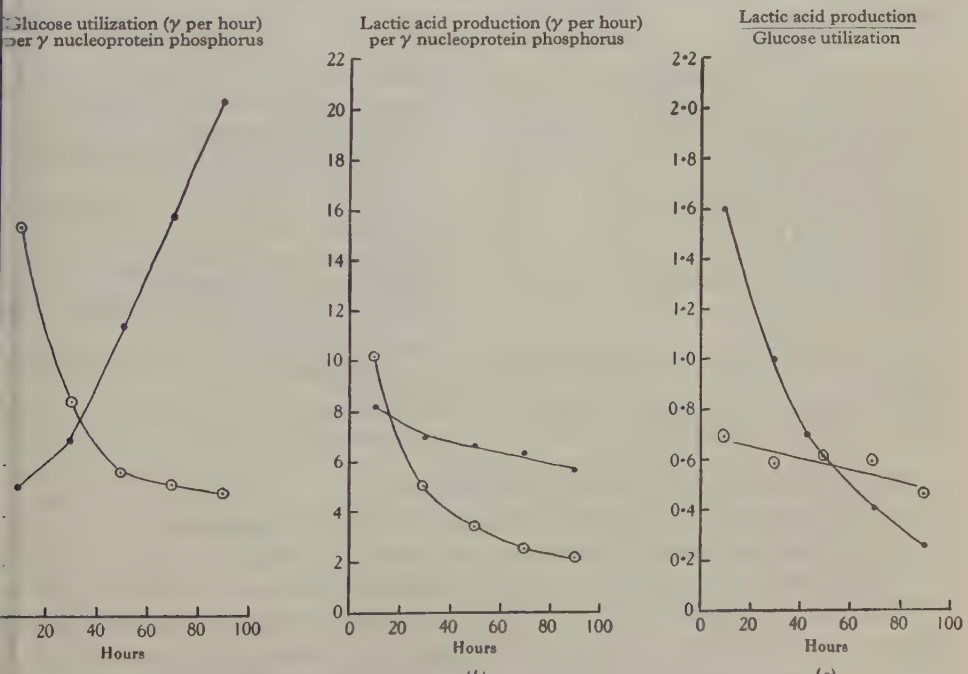


Fig. 6. Glucose utilization and latic acid production by chick osteoblasts.

whole very high in these experiments, which may suggest partial anaerobiosis. The cultures used, however, were small, and they were placed in the thinnest coagulum with constant rotation in about 10 c.c. of air. If all the sugar which disappeared had been oxidized the oxygen tension would have fallen no further than to about 90 mm. of mercury, so that gross anaerobiosis seems unlikely to have occurred, particularly as in the Tyrode medium the ratio falls to a much lower level at the end of the experiment when, owing to the decreased oxygen, lactic acid production should theoretically be higher. It should be remembered, however, that the lactic acid production per unit of nucleoprotein remains higher in Tyrode than in 20% embryo juice. In the early stages, immediately after implantation, both sets of cultures contract and much reorganizing movement must take place as the cells reacquire 'lebensraum', and during this period lactic acid production might well be, and is, higher. Embryo juice causes the cells to migrate much faster so that they restore their optimal distribution much quicker than those in Tyrode. This would suggest that although the glucose consumption might remain at a high level due to the greater activity, the lactic acid production should fall, due to the increased availability of oxygen. The lactic acid production definitely does decrease but the lactic-glucose ratio remains significantly high. In Tyrode solution 'lebensraum' is acquired more slowly and lactic acid production decreases slowly also. The situation is complex, but it does not appear probable that the lactic acid production is correlated only with the availability of oxygen in these experiments. Nor is it correlated with growth rate.

The results with fourth passage osteoblast cultures may be summarized as follows:

Embryo juice	Tyrode
Nucleoprotein phosphorus increases	Nucleoprotein phosphorus remains constant
Glucose utilization is initially very high, but falls quickly to a very low level	Glucose utilization is initially very small but progressively increases
Lactic acid production is initially high but falls quickly to a low level	Lactic acid production is almost constant but falls slightly
$\frac{\text{Glucose utilization}}{\text{Nucleoprotein phosphorus}}$ falls rapidly at first, then very slowly	$\frac{\text{Glucose utilization}}{\text{Nucleoprotein phosphorus}}$ rises steadily and quickly
$\frac{\text{Lactic acid production}}{\text{Nucleoprotein phosphorus}}$ falls rapidly at first, then very slowly	$\frac{\text{Lactic acid production}}{\text{Nucleoprotein phosphorus}}$ falls slowly and steadily, but remains considerably higher than in embryo juice
$\frac{\text{Lactic acid production}}{\text{Glucose utilization}}$ falls slowly from 0·64 to 0·43	$\frac{\text{Lactic acid production}}{\text{Glucose utilization}}$ falls rapidly from 1·6 to 0·26
Cells move rapidly and acquire 'lebensraum' quickly	Cell movement restricted and the culture remains more compact

The chief points which need explanation are:

(1) The rapid fall in glucose consumption in embryo juice.

(2) The rapid rise in glucose consumption in Tyrode.

(3) The continued lactic acid production in Tyrode.

The results suggest that embryo juice determines first a steady uptake of glucose and conversion of much of it to lactic acid during a period of rapid cell movement, accompanied by and perhaps determining (Medawar, 1940), rapid cell

at low and medium concentrations. At high concentrations, however, a similar state of ether saturation, even if only preliminary and local, seems to be reached in the flies from both the acid and alkaline cultures. According to this hypothesis efflux is slower under neutral or alkaline conditions, and hence the acid flies are expected to recover more quickly after exposure to high ether concentration. The following experiments serve to illustrate this.

(*a*) *The influence of exposure to carbon dioxide before and after ether narcosis*

The most convenient means of making the nervous tissue of the flies acid for short periods is by the application of carbon dioxide. As already described, carbon dioxide itself narcotizes the flies if applied in high concentrations. But a combined narcotic effect can be excluded, as in the following experiment.

$T = 23°$ C. 2–3 days old *D. simulans* + flies of both sexes were divided into three batches; one of these was marked by cutting the wing tips of the flies. One batch of the uncut flies was treated for 1 min. with 100 % carbon dioxide and recovered in less than 1 min. The second batch of flies was subjected to 100% carbon dioxide for 1 min., and during recovery the flies were mixed with the third batch and etherized. The average recovery times from narcosis effected by 10·86% ether lasting for $1\frac{1}{2}$ min. were: for seven females narcotized while recovering from 1 min. exposure to carbon dioxide $5·9 \pm 0·8$ min.; for eight males treated in the same way $8·8 \pm 0·7$ min.; for six females narcotized simultaneously as control, $4·1 \pm 0·8$ min.; for seven control males, $4·8 \pm 0·8$ min. The differences are significant in both sexes.

Similar results were obtained with *D. melanogaster* and *virilis*.

The shortening of recovery time from ether narcosis by application of carbon dioxide (well established for man) was described for *Drosophila* by Kalmus (1935). It should be noted that the effect of carbon dioxide on recovery time can also be explained by the action of carbon dioxide in increasing respiration; nevertheless, 100% carbon dioxide should cause apnoea rather than hyperpnoea.

It is possible to explain the quicker recovery from ether narcosis of newly-hatched flies by a more anoxybiotic stage of metabolism at this age (see also the influence of carbon monoxide on p. 251).

Lack of oxygen (vacuum, hydrogen, nitrogen, carbon monoxide) and other factors may also shorten the recovery time, the explanation being that the flies become acid during recovery, especially at interfaces in the tracheal-nervous system. The results of two experiments on the shortening of recovery time by means of carbon dioxide are given below:

$T = 18°$ C. *D. subobscura pl pp op*, 1 day old, narcotized in 10·86% vol. ether for $1\frac{1}{2}$ min. Twelve flies first brought into 100% carbon dioxide for 1 min. recovered after $7·6 \pm 0·77$ min. Eleven flies brought directly into the atmosphere (control) recovered after $12·2 \pm 1·31$ min., the difference of 4·6 min. being significant.

Recovery can also be hastened by putting the etherized flies into a tube with expired air which contains between 3 and 4% carbon dioxide, and it can be shown that neither the draught nor the moisture and increase in temperature resulting from the application of expired air are the cause of this shortening. Application of vaporous hydrochloric, acetic or formic acid, gave results which were not significant. Experiments on the

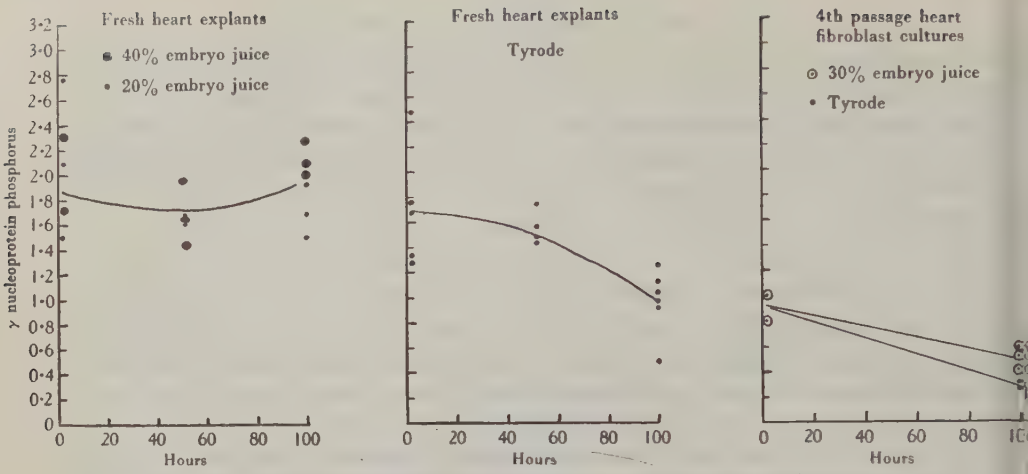


Fig. 7. Nucleoprotein phosphorus content of chick heart tissues in vitro.

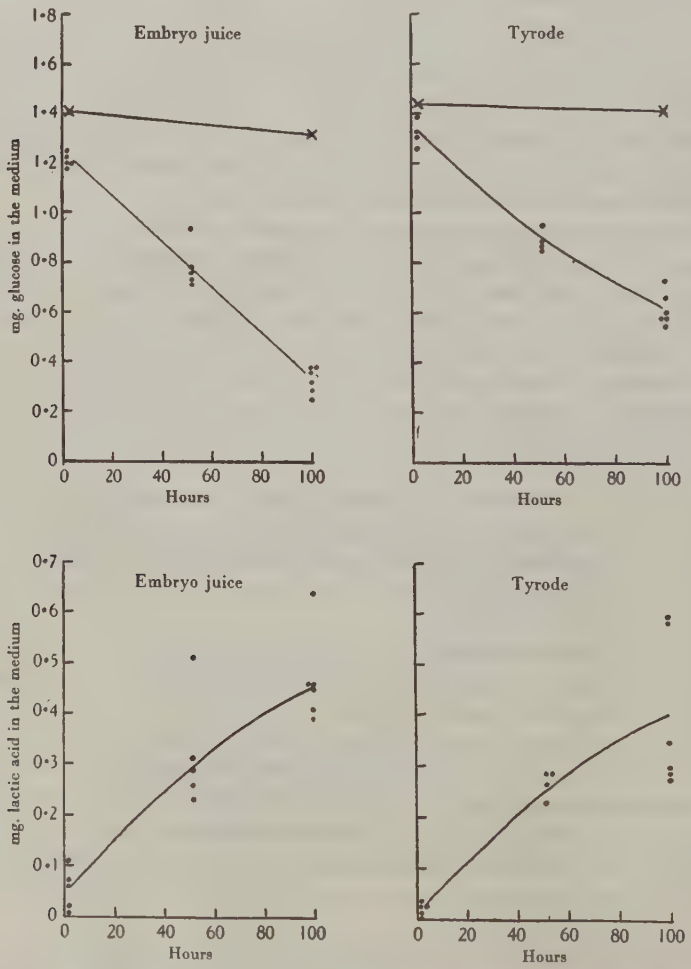


Fig. 8. Glucose disappearance and lactic acid production by fresh heart explants from 9-day chick embryo in embryo juice and Tyrode. Actual concentrations in the medium.

nedium of about 15 % juice and plasma) have a much lower initial nucleoprotein ontent when transplanted into the experimental tubes. The initial amount again ecreases in Tyrode, and the fall is checked with difficulty by embryo juice. Again, s in the case of the osteoblasts, it is significant that nucleoprotein values may remain eady or actually fall in spite of the occurrence of numerous mitoses, for previous

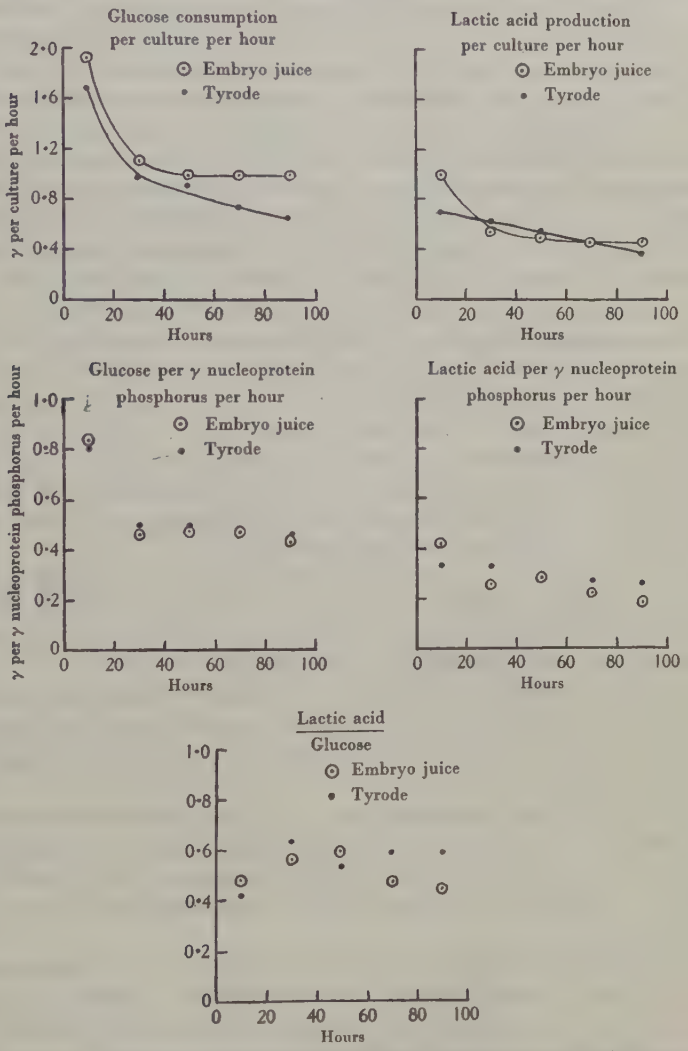


Fig. 9. The glucose consumption and latic acid production of fresh cultures of 9-day-old heart fibroblasts.

ork has shown that embryo heart tissue when freshly explanted, and also after ubculture from embryo juice-plasma media, shows an initial mitotic index of bout 3 % which falls off rapidly to zero in a Tyrode medium and more slowly in 5 % embryo juice. In the latter medium, unless the juice is renewed, mitoses ease on about the fourth day, in the former on the second day.

be ascribed to gene mutation. Similar fallacies are to be expected in all sex-linked mutants. The offspring from a culture may emerge at different times according to the phenotype. In such a case differences in recovery time due to age might be mistakenly ascribed to the effects of gene differences. A serious difficulty is the different behaviour of mutants, races, and species during and after recovery. The definition of recovery as used in this paper (see p. 240) can be applied to all flies, though it does not mean the same in all cases. Immobilization occurs in different ways: *melanogaster*, *simulans*, *pseudoobscura*, and the other small species usually become narcotized gradually, whereas *subobscura*, *virilis* and *funebris* are often immobilized at one stroke. If a mixed batch of *melanogaster* and *virilis* is etherized and then brought back into atmospheric air the *melanogaster* flies are sluggish but not fully narcotized, whereas all *virilis* flies remain motionless. But when the latter are touched with a needle, they start to walk normally, whereas *melanogaster* flies are far from the defined state of recovery for several minutes.

The classification of the degrees of narcosis by most *Drosophila* workers is rather superficial. The desirable degree is defined by immobilization and normal wing position. Flies whose wings are distorted, e.g. dorsally extended in a position similar to that of a butterfly, are usually regarded as dead. This is not always true, even after heavy ether dosages, and does not apply at all to immobilization by carbon dioxide, hydrogen, coal gas, etc., where this position is mostly reversible, as it is after stupefaction with hydrocyanic acid gas (Bliss, 1935). The reversion to the normal wing position occurs suddenly, usually before recovery, but later after heavy dosages.

Differences in the general liveliness of different species, races and mutants also add to the difficulties of comparison. The larger species are much less active and never in constant movement, as is usual with *D. melanogaster*. Wingless mutants obviously cannot flutter as winged individuals often do during recovery.

It is, nevertheless, possible to compare the recovery times of the different *Drosophila* species under specified conditions. If one tries to arrange the species of *Drosophila* according to the time they need for recovery one obtains different sequences dependent on the narcotic, and a different one again for asphyxiation.

Recovery from asphyxiation, achieved by a vacuum or by hydrogen, is significantly quicker in *D. virilis* + than in *D. subobscura*, and quicker in this species than in *melanogaster* or *simulans w* (23° C. 3–5-day-old flies kept together in one bottle for 24 hr. Hydrogen flowing through for 3 min.).

Differences in recovery between *melanogaster or* + and *dp eb* were not constant. There is a striking difference in recovery time after the asphyxiation of *D. virilis sinensis* and *americana*. 3-day-old *sinensis* flies (wings clipped) recovered from 3 min. exposure to hydrogen after $3{\cdot}79 \pm 0{\cdot}62$ min., *americana* flies which had been kept in the same bottle after $5{\cdot}50 \pm 0{\cdot}81$ min.

Great differences of resistance to *hydrocyanic acid poisoning* in *Drosophila* species can be detected by measuring the recovery time. Thus in a single experiment ten *melanogaster or* + flies, ten *virilis* + and 10 *subobscura* + flies were kept together for 24 hr. and then shaken in a hydrocyanic acid killing bottle for 15 sec. The *virilis* flies were not immobilized at all, whereas the median recovery time of the *melanogaster* flies was 4·9 min., and that of the *subobscura* flies more than 20 min.

Recovery from *carbon dioxide narcosis* takes about the same time in the different species of *Drosophila*.

Consistent differences were obtained by simultaneous application of carbon dioxide to *melanogaster or* + and *b vg* or *subobscura* + *subobscura pl pp op*. The multiple mutants of both species recovered significantly slower than the normal flies after 1–15 min. of carbon dioxide narcotization. *D. virilis americana* recovers slightly more slowly than *sinensis*. A stock of *D. melanogaster*, which has been described by L'Héretier & Teissier (1937) as particularly susceptible to carbon dioxide, showed behaviour similar to that of other stocks and had apparently lost this peculiar property.

Recovery from *ether narcosis* may be the same within wide ranges of dosage in distinct species (*D. melanogaster* and *D. simulans*), races (*D. pseudoobscura* A and B) and mutants (*melanogaster* + and eye-colour mutants, *subobscura* + and *int.*). But even in these species after a very high dosage of ether (such as is sometimes applied by geneticists), significant differences can be observed, probably due to differences in the fat content of the flies. Between other species (*melanogaster*, *subobscura*, *virilis*), mutants (especially multiple mutants, e.g. *subobscura* + *subobscura pl pp op*)) and races (*virilis* +, *virilis americana*) significantly different recovery-time curves throughout concentrations could be registered even when conditions were made as alike as possible. Table 4 summarizes some numerical data of recovery times.

Table 4. Drosophila-*recovery time of nine to fourteen flies of each species kept together for* 48 *hr. on* 10% *molasses* (*p*H 6·8). $T = 23°$ C.

1½ min. influx vol. % ether	*virilis* + (*sinensis*) min.	*pseudo-obscura* B min.	*melanogaster or* + min.	*simulans w* min.	*subobscura* + min.	*virilis americana* + min.
6·78	0·30 ± 0·43	1·96 ± 1·10	3·83 ± 1·31	3·88 ± 1·09	5·84 ± 1·18	2·74 ± 0·41
10·86	2·48 ± 1·15	4·93 ± 0·87	6·33 ± 1·19	6·00 ± 1·16	5·84 ± 0·97	5·13 ± 1·90
14·57	7·54 ± 2·01	8·74 ± 1·91	9·62 ± 1·43	9·73 ± 1·35	11·36 ± 1·55	10·10 ± 1·93
23·32	14·05 ± 2·35	15·28 ± 2·70	15·60 ± 2·75	16·72 ± 2·42	18·72 ± 2·84	15·15 ± 2·07

These and similar figures indicate the following order of recovery in the lower range of etherization described: *virilis* + (*sinensis*) recover most quickly, then comes a group consisting of *melanogaster*, *simulans*, *montium* and *pseudoobscura*. Still slower in recovery are *subobscura*, *miranda* and *immigrans*. *Virilis americana* is the slowest to recover, in spite of the fact that *virilis virilis* is the quickest of all. After stupefaction all *sinensis* flies show normal wing position, all *americana* 'butterfly' position (p. 252). Spencer (1940) observed an extreme difference in the stupefaction time of the two races.

Experiments to elucidate the influence of single mutant genes on recovery time have not been very satisfactory. Some preliminary results of experiments are as follows:

(1) Some mutant genes or combinations do not influence the recovery from medium ether dosages of 3–5-day-old flies, e.g. *w*, F_5, *v*, *sc* in *melanogaster*, *int. op.*, *th int* in *subobscura*.

(2) Other genes lengthen recovery *e*, *b* in *melanogaster*, *pl pp op*, *ho* in *subobscura*.

(3) A shortening of recovery time occurs in flies homogeneous for *dp* or *vg* in *melanogaster* and *v* in *subobscura*.

XI. SUMMARY

1. Narcosis and asphyxia in insects can be investigated by measuring the recovery time. This and other terms are defined, and suitable criteria of recovery are given.

2. Simple techniques for the etherizing and gassing of *Drosophila* batches are described and the validity of the quantitative results obtained is shown.

cultures to compare with osteoblasts, but such data as have already been obtaine indicate that the heart fibroblasts continue to react in very much the same wa and that the nucleoprotein content continues to decrease in Tyrode.

Another point of interest in connexion with the nucleoprotein phosphorus dat is the one upon which comment has already been made, namely that increasing th strength of the extract increases the rate of building of the new nucleoprotein, bu that in these experiments a limit is set to the total increase, probably because of th shortage of some essential material originally present in the plasma clot. It i significant in this connexion to recall once again Laser's experiments (1933*a*) i which he found that cultures in serum and plasma put on more weight in 4 day than did similar cultures in embryo extract and plasma, although the latter grev to much greater areas and are known from other methods to show far more ce divisions. This increase in weight in serum was almost certainly not due in th main to increase in the number of cells, since serum has been shown not to increas the number of cell divisions. Embryo juice perhaps determines nuclear synthesis while plasma and serum feed the cells and produce cytoplasmic growth an intercellular matrices. For prolonged growth both constituents of the diet ar required. This may be compared with the growth-promoting action of embryo juic on cells growing in proteose solutions. Measurements of glucose consumption an lactic acid production by cultures growing in serum, in order to ascertain whethe they are correlated to nucleoprotein or to dry weight, would complete the picture

Turning now to the glucose consumption by tissues in vitro there is little doub that embryo juice hastens the uptake of sugar by osteoblasts and to a much les extent by heart fibroblasts and that much of this sugar is transformed into lacti acid. Some of the extra consumption is almost undoubtedly due to the greate cell movement. This latter is greatly increased at once when embryo juice is adde to a culture quiescent in Tyrode and plasma. Tissues with high growth energy like fresh heart explants from young chicks, reorganize quickly and spread thei cells out into the medium without delay as soon as they are planted. The sam occurs when tissues are planted into a medium containing embryo juice, but ol colonies planted in plasma and Tyrode take longer for their cells to spread ou The glucose consumption and lactic acid production appear to follow the sam lines, with the exception that the glucose consumption of cultures in plasma an Tyrode gradually mounts, although the lactic acid production does not ris proportionally. In general, then, glucose consumption, when accompanied b corresponding lactic acid production rises with increased cell movement, and seems probable that increased cell movement is a necessity for the growth fibroblasts by cell division (Medawar, 1940).

The increasing glucose consumption in Tyrode is unexplained. The glucos may be stored, but glycogen is not a conspicuous feature of cells in vitro, excep incidentally of heart muscle, and in these experiments heart tissue is the very on which does not show increasing glucose utilization. Cells in plasma and Tyrod do not become laden with fat; in fact they remain conspicuously hyaline. Possibl glucose metabolism suffers and some intermediate step in the formation of lact

is inhibited so that glucose disappears without a corresponding lactic acid mulation. Another possibility is that more glucose may be directly oxidized rbon dioxide and water, but it is difficult to see why carbohydrate metabolism ld be so greatly increased, unless perhaps the energy supply from other sources iling. Finally, differentiation may be occurring under the more stagnant itions of life in plasma and Tyrode, and the glucose may be being used for manufacture of some essential differentiation product, glucoproteins for mple, although against this it may be noted that the formation of argyrophil s, a rather similar process of differentiation and an early sign of its occurrence, takes place freely when embryo juice is present in the medium.

Lactic acid formation in general follows the glucose consumption, except in the nce just cited, namely that of osteoblasts in Tyrode. It is noticeable that with oblasts, when much growth is occurring in embryo juice, the ratio of lactic acid duced to glucose consumed is high. As the growth rate falls off in the course ne 4 days of the experiment the ratio gradually falls also. This concept of the elation of high growth rate and high lactic-glucose ratio is also consistent with figures found for osteoblasts in Tyrode solution although other factors enter the situation there and modify the details. Tyrode cultures become much more avagant of carbohydrate than those in embryo juice. This is curious in that, only is cell movement very much reduced, but also because one of the observed cts of embryo juice is an initially greater uptake of sugar by the tissue. It gests that embryo juice, in spite of its immediate action in promoting glucose ake, a process which may be correlated with increased cell movement, makes cells economical of carbohydrate, perhaps by favouring protein or fat metabolism, it is possible that the high proportion of lactic acid may indicate its origin from e other source than sugar. On the other hand, the absolute amount produced unit of nucleoprotein phosphorus is markedly smaller than in Tyrode which n observation more in favour of a feebler oxidation of the carbohydrate in the sence of embryo juice than its origin from another source; so also is the constancy he ratio in spite of varying glucose uptake.

Returning once again to the theme of high lactic acid production and growth only indication in the data collected in these experiments pointing in that ction is the high lactic-glucose ratio of growing osteoblasts. Heart and skin not however show this. There is therefore ample confirmation of the work of enblum & Chain and of Boyland & Boyland in that there appears to be no ential correlation between high growth rate and high lactic acid production.

SUMMARY

1. The glucose consumption of osteoblasts growing in a medium of plasma and bryo juice has been compared with that of the same cells in plasma and Tyrode dium. Embryo juice causes an initial increased glucose consumption which later s below the level characteristic of a Tyrode-plasma medium.

2. A modification of a method of measuring the nucleoprotein phosphorus tent of tissue cultures is described and among other data obtained the changes

The second method, a variation of the first, consisted in clamping the crayfish at an angle of about 45 degrees, ventral side up. The clamp is pivoted on the cut chitin of the cervical groove and the motion of the heart is transmitted downwards. The kymograph records obtained are the reverse of those obtained with the other methods. This method has been found particularly advantageous in the investigation of the effects of drugs, in which rapid changes in the perfusion fluid are desired.

In the third method, which was used in the study of the effect of the stimulation of the central nervous system, the crayfish is clamped in a horizontal position. The heart is in a dorsal position and, as in the second method, the clamp is made to pivot on the cut chitin of the cervical groove. The perfusion fluid level is adjusted so that the oesophageal commissures are just covered, in which case the fluid does not quite reach the heart. Perfusion of this organ is, therefore, less perfect than in the other methods. In experiments of rather short duration, however, a comparison of this method with the first one did not show any significant difference. This method makes possible isolation of fibres in the oesophageal commissures (Wiersma, 1938).

The influence of the frequency of stimulation has been determined with two thyratron stimulators. Stimulations of 15 sec. duration have been regularly used and, in the tables and records, the number of beats are given for that interval unless specific times are given. The phenomena reported here have been consistently observed and the total number of preparations has been large.

RESULTS

The effect of stimulation of the peripheral inhibitors

By evisceration, the course of the pair of peripheral inhibitors from the suboesophageal ganglion to the heart is exposed for most of its length. The nerves enter the main body cavity through an easily recognizable square hole, situated above the suboesophageal ganglion (Text-fig. 1). From there they take a latero-dorsal course running along the ridge of chitin to which the flexor muscles in the ventral part of the thorax are attached. Near the point where the most lateral heads of this muscle group are attached, the course of the nerves becomes more caudad and crosses the surface of the extensor muscles in the thorax. These nerves probably correspond to the second superior nerves from the suboesophageal ganglion which Keim (1915) described for *Astacus*.

The nerve is easily freed from its attachment to the chitinous ridge, and can then be selectively stimulated by raising it on electrodes or by pressing a pair of electrodes against it.

In a fresh preparation, stimulation of one nerve with frequencies of about 40 per sec. normally results in a complete interruption of the heart beat. As with stimulation of the vagus of the vertebrates, such stoppage is not permanent, but is followed by a partial escape. In some cases a stoppage of several minutes was obtained. The onset of complete inhibition is either instantaneous, or one reduced beat may occur (Pl. 1, fig. 1:60), depending on the moment of the beat cycle when the stimulation is started. The heart normally starts beating immediately after the end of stimulation, usually at a frequency slower than normal, speeding up rather gradually (Pl. 1, fig. 1:60). This after-effect will be discussed in more detail later.

Variation in stimulus strength at a frequency which causes complete inhibition shows a close adherence to an all-or-none relation. Stimuli within the very narrow range between a strength resulting in complete stoppage and one without any influence may show an increase in the number of escape beats with decrease in stimulation strength.

ON PROPERTIES OF THE RED CELL GHOST

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(With Two Text-figures)

I. VOLUME AND SHAPE CHANGES DURING HAEMOLYSIS AND "REVERSAL OF HAEMOLYSIS"

HEN the mammalian red cell is put in a hypotonic solution, it undergoes an increase volume accompanied by changes in shape. If the solution is sufficiently hypo-ic, it swells until it reaches its "critical area" substantially the same as the area he original biconcave disk (Ponder, 1937; Castle & Daland, 1937). The cell then molyses, losing salts and haemoglobin; the tonicity inside becomes equal to that he surrounding fluid, but the haemoglobin concentrations inside and outside do come into equilibrium, an excess of pigment ("residual haemoglobin") re-ining inside. If the hypotonic system is restored to isotonicity by the addition of Cl, "reversal of haemolysis" occurs. The almost transparent haemolysate be-mes opaque, and under certain circumstances the ghosts can be centrifuged down moderate speeds. Looked at from an osmotic point of view, the volume which y might assume is indeterminate; ghosts completely permeable to salts, as well to water, would not shrink at all, ghosts impermeable to salts would shrink to a all fraction of their volume, and ghosts permeable to water but slowly permeable salts (as these are, Davson & Ponder, 1938) might take up any volume between two extremes. If the volume and shape changes are followed, some interesting d unexpected points emerge.

The volume concentration of ρ of a suspension of non-conducting red cells or osts can be found from the expression

$$\rho = (r_1/r_2 - 1)/(r_1/r_2 + 1/X),$$

which r_1 and r_2 are the resistances of the suspension and of the suspension fluid spectively, and X a "form factor", which depends on the shape of the cells. For heres, $X = 2{\cdot}0$, and for rabbit cells in the discoidal form, $X = 1{\cdot}2 \pm 0{\cdot}1$, but even considerable change in form has relatively little effect on the volume concentra-ons, e.g. if the cells were spheres instead of disks, ρ would be only 10% too high.

The difficulty is to obtain the suspension fluid free of ghosts, but this can be ne either by high speed centrifuging (5×10^4 times gravity) or by filtration. entrifuging is the more convenient, but its efficacy in completely freeing the spension fluid from ghosts varies with species, and also with conditions which are t completely defined. For removing the ghosts by filtration, I use Zsigmondy embrane filters. These are flat membranes with the stiffness of parchment, and are ed like a piece of filter paper in a Buchner funnel connected with a flask and a ction pump. A "medium-grade" filter, with a pore size of from 0·1 to 1·0μ,

filters out rabbit watery ghosts completely. Unfortunately the ghosts clog up the pores of the membrane, and so filtration is slow; for this reason I use a micro-conductivity cell which requires only 0·3 c.c. of fluid; I also use a micro-filtration apparatus, the diameter of the perforated area of the Buchner funnel being only 11 mm. Micro-membrane filters can be made from the Zsigmondy membranes by cutting out 15 mm. circles with a cork-borer, and the membrane can be sealed into the micro funnel by running a vaseline-beeswax mixture round its circumference after it is pressed into place against the perforated area. A representative sample of the supernatant fluid is obtained if the first sample of the filtrate is rejected and if the micro-filters are pressed between sheets of filter paper just before use.

The way in which the volume and shape changes during haemolysis and "reversal of haemolysis" are followed is best described by giving an account of a typical experiment.

(*a*) *Lysis.* The cells of 20 c.c. of defibrinated rabbit blood are washed twice, and made up to a volume of 20 c.c. with 1 % NaCl. The resistance of the suspension, measured in the micro-conductivity cell immersed in a constant temperature water bath, is $194{\cdot}0\omega$, and that of the supernatant fluid (obtained by gentle centrifuging) is $96{\cdot}5\omega$; using a form factor of 1·25, this gives $\rho = 0{\cdot}359$ (percentage volume = 35·9). To 6 c.c. of water, 1 c.c. of the suspension is added; if no change in the cell volume were to occur, this seven-fold dilution would reduce the value of ρ to 0·359/7, or 0·052 (percentage volume = 5·2). In actuality, the cells swell to their critical volume (about 170 % of their initial volume) and then haemolyse, and it has already been shown that just before haemolysis they assume the form of spheres (Ponder, 1937; Castle & Daland, 1937).

The resistance of the haemolysate is $780{\cdot}3\omega$ and that of the supernatant fluid, obtained by high speed centrifuging, is $703{\cdot}7$; this gives $\rho = 0{\cdot}058$ if the ghosts are disks and we use $1/X = 0{\cdot}8$, or $\rho = 0{\cdot}069$ if the ghosts are spheres and we use $1/X = 0{\cdot}5$. Microscopical examination leaves no doubt that the ghosts are discoidal, although somewhat deformed and clumped together, and so we select the figure, $\rho = 0{\cdot}058$. If the ghosts had the same volume as the cells from which they were derived, ρ would be 0·052; if the volume of the original discoidal cells in 1 % NaCl is denoted by 100, the volume of the discoidal ghosts in the hypotonic haemolysate (tonicity = about 0·14) is 111, and the increase in volume is only 11 %. Taking into account the fact that before haemolysing the cells were spheres with a critical volume of about 170 %, and that the errors in the volume measurements may amount to 10 %, the conclusion is that the ghost derived from the red cell as a result of haemolysis in a hypotonic solution shrinks back to the original volume of the cell, at the same time becoming discoidal once more.[1]

[1] Using conductivity methods, Fricke & Curtis (1934) came to the conclusion that when red cells are haemolysed with from 3 to 19 parts of water the volume of the ghosts is from 1·45 to 1·65 times that of the original cell, with an average value of 1·54. In making the computations they assumed that the ghosts are spheres instead of disks. Using the proper form factor, $1/X = 0{\cdot}8$ instead of 0·5 reduces their average value to 1·34. This is certainly greater than the figure 1·11 (or 111 %), which I have obtained and think of as being the same as unity, but Fricke's methods of computation are indirect. At all events, considering that Fricke and Curtis's tonicity must have been in the neighbourhood of 0·25, their results are not essentially opposed to mine.

(*b*) "*Reversal of haemolysis.*" The rapid conductivity changes which accompany ersal of haemolysis are best studied in a larger conductivity cell, in which the tents can be thoroughly mixed at constant temperature. As before, haemolysis is duced by adding 1 c.c. of a suspension of washed rabbit red cells, in this case h $\rho = 0{\cdot}307$, to 6 vol. of water, and transferring 3·5 c.c. of the haemolysate to the ductivity cell. The resistance of the haemolysate is $588{\cdot}8\omega$, and that of the ernatant fluid $542{\cdot}0\omega$, giving $\rho = 0{\cdot}045$ as compared to the expected 0·307/7, or 44. To the contents of the conductivity cell 0·5 c.c. of 7% NaCl is added, with rough mixing. "Reversal of haemolysis" takes place, and the resistance falls hin a few seconds to 86ω. Thereafter it steadily increases, at first rapidly and n more slowly, to become steady at $89{\cdot}3\omega$ after about 10 min. The resistance of supernatant fluid was $82{\cdot}7\omega$, giving $\rho = 0{\cdot}042$, instead of the expected 0·038 07/8), which would be found if the ghosts were discoidal and had the same ume as the original volume of the cells from which they were derived. Micro- pical observation shows them to be somewhat deformed disks, and the dis- pancy ($\rho = 0{\cdot}042$ instead of 0·038) is within the limits of error of the method at se small values of ρ. The conclusion is that after reversal of haemolysis is com- te and the system has reached a steady state, *the volume of the ghost is substantially same as the original volume of the red cell.*

Immediately after the restoration of isotonicity, however, the resistance fell to ω, and, assuming that the resistance of the suspension medium was $82{\cdot}7\omega$ at this ge also, the volume concentration of the ghosts must have been only 0·027, i.e. addition of the 0·5 c.c. of 7% NaCl brought about a shrinkage of the ghosts to out 65% of their volume. This shrinkage is followed by a slow swelling until the istance of the system was $89{\cdot}3\omega$, and the volume of the discoidal ghost the same the original volume of the red cell, and thus the cycle of volume and shape anges is completed. The shrinking of the ghost on restoration of isotonicity is to expected because the ghosts, at equilibrium in the haemolysate at a tonicity of out 0·14, are not freely permeable to the added salt; their subsequent swelling to ir original volume may result from their being slowly permeable to ions such as and K (Davson & Ponder, 1938).

These observations (illustrated simply in Fig. 1) bring out two points. The first hat when there are no osmotic forces acting upon it, the watery ghost tends to e up the volume and shape of the red cell from which it was derived. This ggests that both have some kind of structure, which, in the absence of greater rces, determines the shape and the volume; so far as these experiments go, this ucture might be an internal meshwork or a sufficiently organized surface mem- ane (cf. Norris, 1939). The second is that the ghost does not shrink as much as ght be expected when the tonicity of the fluid surrounding it is increased. In the regoing experiment a shrinkage to only about 65% of the volume of the ghost was served when the tonicity was suddenly raised from 0·14 to 1·0, whereas a body permeable to the added salt would shrink to about 14% of its volume. That the rinkage is so relatively small may be in part accounted for by the fact that the ost is slowly permeable to the ions of the added salt, but it may also be due to

some structure, such as an internal gel, preventing the volume decreasing beyond a certain point. It may not be a coincidence that a shrinkage to 65 % of the original volume corresponds to an abstraction of about half the cell water, which other experiments have shown to be the greatest amount which can be removed by osmotic means (Gough, 1924; Krevisky, 1930; Ponder & Saslow, 1931).

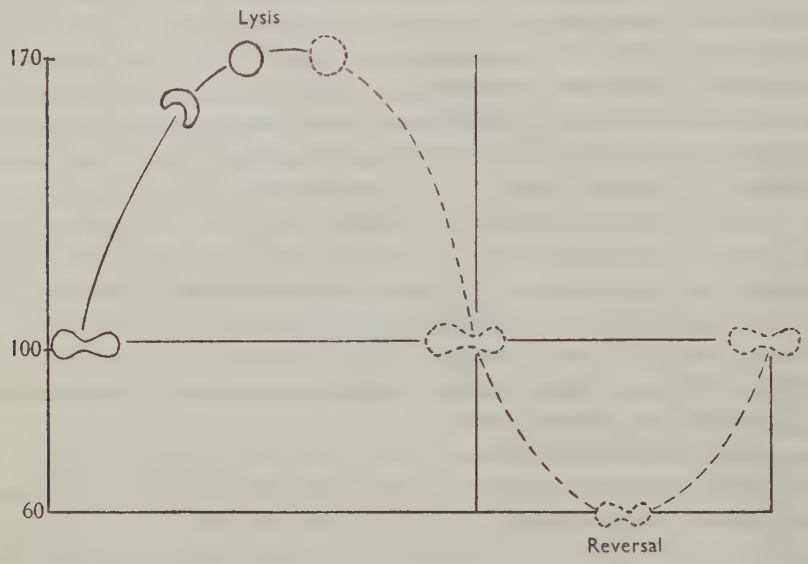

Fig. 1.

While these experiments show that the watery ghost tends to take up the same volume as that of the red cell from which it is derived, it does not seem possible to haemolyse red cells by water, throw down the ghosts at ordinary speeds (about 10^4 times gravity), and obtain a 100 % yield by volume. Further, if the ghosts are washed, even with CO_2-saturated water or with 0·1 % NaCl (the latter makes it possible to follow the volume concentrations by conductivity), each successive yield is smaller than the one before it, and the decrease in ρ usually being about 10 % per washing. The loss is still greater if 1 % NaCl is used for washing. This diminution in volume might be accounted for in three ways: (*a*) All the ghosts might not be thrown down by the centrifuge, (*b*) a decrease in the volume of the individual ghost might occur, or (*c*) some of the ghosts might cease to be non-conductors of the electrical current. There is no evidence in favour of the last possibility, and meantime I am inclined to attribute the loss to the ghosts being incompletely thrown down. It is true that there are some features about the microscopical appearance of the watery ghost (see § III) which make one consider possibility (*b*), especially as the cell, in becoming a ghost, loses haemoglobin. It will be shown in the next section, however, that the cell does not lose as much pigment during haemolysis as one might think.

II. RESIDUAL HAEMOGLOBIN

When the red cell haemolyses in a hypotonic medium and becomes a ghost, the concentrations inside and outside become equal, but the haemoglobin concentrations usually do not. For the sake of clearness it is convenient to think of the haemoglobin associated with the ghost, or the "residual haemoglobin", as made up of two quantities. The first is that which the ghost would contain by virtue of an equilibrium between the pigment inside it and that outside it in the suspension used; this will be referred to as the "equilibrium haemoglobin". The second is that will be referred to as the "surplus haemoglobin", the presence of which results in the ghost containing an excess of pigment as compared with the suspension fluid. So residual haemoglobin = surplus haemoglobin + equilibrium haemoglobin.

(1) *Residual and surplus haemoglobin.* To measure these, one suspends the washed cells of 10 c.c. of rabbit blood in about the same volume of 1 % NaCl, and finds the volume concentration by conductivity. The cells are then haemolysed by adding them to 6, or 12, or 18, or 24 vol. of water. After standing for about half an hour, reversal of haemolysis is brought about by adding 10 c.c. of a NaCl solution of sufficient concentration to restore isotonicity. The ghosts are centrifuged down after about 30 min., and the conductivity of samples of the layer of ghosts and of the supernatant fluid is measured; this gives v, the volume concentration of the ghosts. 5 c.c. of the supernatant fluid is added to 50 c.c. of 1 in 1000 saponin, and 1 c.c. of the layer of ghosts is treated similarly; after standing for some hours and after vigorous centrifuging, the haemoglobin content of each is found with the Zeiss Pulfrichphotometer at a mean wave length of 4300 A. In general, the concentration of haemoglobin in the solution prepared from the ghosts is greater than that in the solution prepared from the supernatant fluid; the photometer measures F, the ratio of these two quantities, so

$$C_1/C_2 = (F + v - 1)/v,$$

where C_1 is the residual haemoglobin of the ghosts, and C_2 the haemoglobin of the fluid surrounding them.

The average values of C_1/C_2 obtained in a series of 12 experiments with different volumes of water, W, used for haemolysing the cells are shown in Table 1.

Table 1

W, vol.	6	12	18	24
C_1/C_2	3·8	1·66	1·58	1·24

If only equilibrium haemoglobin were present, C_1/C_2 would be unity, so the surplus haemoglobin is measured by $C_1/C_2 - 1$. Plotting this against $1/W$, which is a measure of the concentration of haemoglobin in the suspension fluid, we find that the surplus haemoglobin is roughly proportional to the haemoglobin concentration surrounding the ghosts. The range of concentrations being small, this relation is compatible with the surplus haemoglobin being adsorbed by the

"fixed framework" of the ghost, and this raises the question of the nature of the adsorbent, which might be the surface structure, or a more extensive internal network.

It is not possible to answer the question with certainty, but the following computation gives an idea of the possibilities. The maximum value for the surplus haemoglobin is about twice that in the suspension fluid of the haemolysate, and the concentration of this is one-eighth of that in the whole blood of the rabbit, or $0{\cdot}125 \times 107$ g. Hb/l. With a molecular weight of 64,000 for haemoglobin, this corresponds to $1{\cdot}22 \times 10^{20}$ mol./l., and the surplus haemoglobin is twice this, or $2{\cdot}44 \times 10^{20}$ mol./l. Regarding the haemoglobin molecule as a sphere with radius 19 A, the projected surface of each molecule would be 1130 A^2, and the total area would be $2{\cdot}76 \times 10^{23}$ A^2. Since the total surface of the red cells contained in a litre of rabbit blood is about $6{\cdot}6 \times 10^{22}$ A^2, the surplus haemoglobin would form a layer only about 4 molecules deep at the surface of the ghosts, and so might all be adsorbed on the surface ultrastructure.[1]

Such an adsorption by the fixed framework is compatible with two easily demonstrated properties of the surplus haemoglobin: it is very difficult, if not impossible, to remove entirely by washing with water or with NaCl, but it is readily eluted by substances such as saponin. The quantity of surplus haemoglobin, furthermore, is slightly greater at low temperatures than at high, although the differences which I have been able to find are small ($C_1/C_2 - 1 = 0{\cdot}63$ at 3° C.; 0·52 at 25° C.; 12 vol. water).

(2) *Equilibrium haemoglobin.* This quantity of pigment is (by definition) in equilibrium with the haemoglobin in the fluid surrounding the ghost, and can be removed by repeated washing. From this it follows that the membrane of the ghost is permeable to it, and this suggests the experiment of trying to introduce haemoglobin into the ghosts by adding it to the fluid surrounding them.

Relatively pigment-free ghosts[2] are prepared by haemolysing the cells of 10 c.c. of rabbit blood with 6 volumes of water; CO_2 is bubbled through the haemolysate for a few minutes until the *p*H reaches about 5·5, and the ghosts are then easily thrown down by centrifuging at moderate speeds. They contain a considerable amount of residual (surplus + equilibrium) haemoglobin, but three or four washings with 0·1 % NaCl saturated with CO_2 removes most of this, and one is finally left quite a good yield of tan-coloured ghosts, the volume concentration, ρ, of which is found by conductivity. For a good experiment ρ should exceed 0·5. At least 5 c.c. of the supernatant fluid from the last washing is set aside; this is usually slightly coloured with pigment in the same concentration as the equilibrium haemoglobin, and will be referred to as the "weak haemoglobin solution".

A "strong haemoglobin solution" is made by haemolysing packed rabbit red

[1] If we regard the haemoglobin molecule as an oblate spheroid or as a cylinder, which are more likely shapes than that of a sphere (cf. Neurath, 1939), the results are not so good. Assuming a spheroid with major axis 158 A and with minor axis 31·6 A, the projected area of each molecule might be as much as 3900 A, and so the number of layers at the surface might be, not 4, but about 14.

[2] The method for preparing entirely haemoglobin-free ghosts will be described by A. J. Parpart, who has kindly communicated it to me and to R. F. Furchgott.

lls by freezing and thawing, and this is kept frozen in the refrigerator until re-ired, when it is diluted 1 to 10 with 0·1 % NaCl. To 2 c.c. of the suspension of osts is added 2 c.c. of strong haemoglobin solution; after mixing and standing for rying lengths of time, the ghosts are thrown down and the supernatant fluid X moved for matching against two standards. The first of these, CP, contains the ncentration of haemoglobin which would result in the unknown X if the pigment npletely penetrated the ghost, and is made by adding 2 c.c. of weak haemoglobin ution to 2 c.c. of strong haemoglobin solution. The second, NP, contains the ncentration which would result if there were no penetration, and is made by ding $2(1-\rho)$ c.c. of weak haemoglobin solution to 2 c.c. of strong haemoglobin ution. These standards correspond to complete or zero penetration, of course, ly if there is no adsorption of haemoglobin by the ghosts.

The two standards, CP and NP, and the unknown X, are diluted 1 to 10, and e haemoglobin concentrations are measured with the colorimeter.[1] There are partures from Beer's and Lambert's laws, but a good enough measure of the ent of penetration of haemoglobin into the ghost, in the absence of any adsorp-n, is provided by

$$P = 100\,(X-NP)/(CP-NP),$$

ere X, CP, and NP are the readings corresponding to the unknown and the two ndards respectively. If there is no penetration (and no adsorption), $P=0$; if netration is complete and there is no adsorption, $P=100$; degrees of partial netration are represented on a percentage scale.

Unfortunately attempts to measure the penetration of haemoglobin in this way e complicated by adsorption of the added pigment by the ghosts, as is shown by e fact that X is sometimes greater than CP, so that P becomes greater than 100 Table 2, Exp. 5). Indeed it would be possible to attribute *all* of the loss of the

Table 2

Exp. no.	T (min.)	P (%)
1	60	76
2	300	100
3	10	34
	60	55
4	10	42
	180	59
5	10	127
	180	210

emoglobin from the suspension fluid to adsorption; one must remember never-eless that the membrane of the ghost is permeable to haemoglobin to some degree, cause the equilibrium haemoglobin can be washed out, and so one would expect at a penetration of the pigment would contribute to some extent to the value of P. here does not appear to be any way at present in which the penetration process and

[1] Measurements of the concentrations with the colorimeter are much more satisfactory than terminations of the extinction coefficients with an instrument such as the Stufenphotometer. This partly due to the difficulty in working visually at 4300 A.

the adsorption process can be separated, and so I give in Table 2 a number of values of P which have been obtained, but with an appreciation of the difficulty which one meets in interpreting them. The quantity of haemoglobin which disappears from the suspension fluid is certainly not too great to be adsorbed, for it would not cover more than about one-tenth of the surface of the ghosts with a monolayer.

So the result of hypotonic haemolysis is a permanent damage to the cell membrane, as shown by the permeability to haemoglobin (certainly in the direction inside—outside, probably in the direction outside—inside) and to cations (Davson & Ponder, 1938). Fricke & Curtis (1934), moreover, have pointed out that there is a change in the frequency dependence of the impedance of the membrane after haemolysis, which is a further indication of permanent injury, and it will now be shown that when the cell becomes a ghost it is permanently altered in even another way, for it no longer exhibits disk-sphere transformations.

III. SHAPE AND SHAPE TRANSFORMATIONS

The shape of ghosts, and their shape transformations, are best described by a number of observations, with explanatory notes attached.

(1) *Watery ghosts after reversal of haemolysis.* These ghosts regain their original volume and shape (see § I). Although they are somewhat deformed, they are unquestionably biconcave disks. They tend to stick together in clumps, and, as has been shown in § II, they retain some residual haemoglobin. The sticking together makes it difficult to see them on edge. Unlike the original red cell, the ghost will not form a sphere between two closely applied glass surfaces, nor will it sphere on the addition of lecithin. The addition of saponin or rose bengal, however, may sometimes cause sphering, and causes subsequent lysis, with the loss of such residual haemoglobin as may be contained.

(2) *Watery ghosts precipitated by* CO_2. When watery ghosts are thrown down after bubbling CO_2 through the haemolysate, it is possible to wash them repeatedly with CO_2-saturated water or dilute saline without much loss, whereas watery ghosts produced by reversal show a rapidly diminishing yield on washing. It may be because it is possible to wash them so many times that watery ghosts prepared with CO_2 are so much thinner and more tenuous than watery ghosts prepared by reversal. The ghosts are good disks and can easily be seen on edge, especially if a little rose bengal is added. No sphering can be observed between slide and slip, with lecithin, with saponin, or with rose bengal.

(3) *Saponin ghosts.* Ghosts produced by the addition of sufficient saponin to render them completely permeable (as judged by conductivity measurements) are irregularly crenated, but substantially spherical masses. Being substantially spherical, nothing of the nature of disk-sphere transformations can be observed.

(4) *Freezing and thawing ghosts.* These are roughly circular masses, about 6μ in diameter. They tend to agglutinate, and, being roughly spherical, show no disk-sphere transformation. They are certainly not "fragments" or "droplets", as is so often implied in the literature.

In the case of ghosts produced by saponin or by freezing and thawing, the "shape mponent" apparently is destroyed completely, so that the cells become roughly herical. (In the case of lysis by sufficient saponin, the "permeability component" ist be destroyed also, for the cells appear to be conductors.) In the watery ghost, e "form component" seems to be preserved, for the ghosts are biconcave disks; e surface, however, must have undergone a modification, for the typical disk- here transformations no longer take place.

In the present state of our knowledge, it is not worth speculating as to what this dification may be. This paper pretends to do nothing more than to describe some the complex properties of the red cell ghost. A description of other related perties, also difficult to understand, are to be found in a paper by R. F. Furch- tt (1940).

SUMMARY

This paper is concerned with certain properties of the mammalian red cell ost. The principal conclusions are:

1. When red cells are haemolysed by water they increase in volume, changing eir shape and becoming spheres. If the degree of hypotonicity is sufficient they emolyse in the spherical form, but quickly resume the form of disks, even in potonic solutions. If the haemolytic systems are rendered isotonic by the addition salts, the cells shrink temporarily, but finally assume their original volume and ape. It seems that when there are no osmotic forces acting on it, the watery osts tend to take up the volume and shape from the red cell from which it was rived.

2. When a red cell haemolyses in a hypotonic medium and becomes a ghost, a nsiderable amount of haemoglobin is retained, perhaps by adsorption. It is ubtful whether this adsorption could be on a purely surface structure; to account r the results we might have to postulate an internal structure in addition.

3. After haemolysis by water, red cells will not form spheres between slide d coverslip, nor by the addition of lecithin. If the watery ghosts are made by ecipitation with CO_2, no sphering can be observed between slide and slip, with cithin, with saponin, or with rose bengal. In the case of the watery ghost, the orm component" seems to be preserved, for the ghosts are biconcave disks; the rface, however, must have been altered, for disk-sphere transformations no nger take place.

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THE RESPIRATORY FUNCTION OF THE HAEMOGLOBIN OF THE EARTHWORM[1]

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(With Two Text-figures)

It was claimed by Davy as early as 1862 that the haemoglobin in the blood of the earthworm has a respiratory function, but subsequent studies have given conflicting results. In the more recent investigations advantage has been taken of the fact that haemoglobin has a greater affinity for carbon monoxide than for oxygen; the oxygen consumption of normal worms is compared with that of worms whose haemoglobin has been put out of action by saturation with carbon monoxide. Using this method, Jordan & Schwarz (1920) concluded that haemoglobin functions as a transporter of oxygen only at low pressures of oxygen (23–30 mm. mercury).[2] Dolk & van der Paauw (1929) criticized this work on the grounds that the pure carbon monoxide to which the worms were subjected for some hours might have affected the respiratory enzymes as well as the haemoglobin, and that because of individual variation it was not possible to compare directly the oxygen consumption of normal and carbon monoxide-treated worms. They repeated the work with modifications, and again found that haemoglobin was functional as an oxygen transporter only at low pressures of oxygen, below 57 mm. mercury. Their work was done on narcotized animals, and was therefore criticized by Thomas (1935), who, moreover, considered that the conclusions drawn by Dolk & van der Paauw were not justified by the experimental data they published. Thomas reinvestigated the matter, and came to the conclusion that haemoglobin has no respiratory function at oxygen pressures lower than 114 mm., for the oxygen consumption of normal and carbon monoxide-treated worms was the same. Above 114 mm. oxygen pressure the oxygen uptake of normal worms rose sharply, while that of carbon monoxide-treated worms remained constant, but Thomas did not attribute this difference in behaviour to haemoglobin, and one is left with the impression that in the earthworm haemoglobin is functionless as an oxygen transporter at all pressures of oxygen. Thomas's results must, however, be accepted with reserve, for he gives data of experiments on only two normal worms and three carbon monoxide-treated worms.

[1] A preliminary account of these results has been published elsewhere (Fox, 1940).

[2] In the paper of Jordan & Schwarz, and in some other papers subsequently discussed, the amounts of oxygen in the respiratory medium are given as percentages, the gas mixtures being at atmospheric pressure. In order to make comparison with other results easier, I have in this paper expressed these percentages as pressures of oxygen in mm. of mercury.

1938 a brief report was published by Krüger of work on the function of earthorm haemoglobin. Krüger found that haemoglobin transports oxygen at atmoheric and also at lower partial pressures of oxygen; for the oxygen uptake of rbon monoxide-treated worms was lower than that of normal worms at pressures 152, 114, 76 and 38 mm. Unfortunately in this report the experimental method ed is not described and no detailed data are given (for instance, the actual rates oxygen consumption are not stated), so it is impossible to evaluate the evidence which Krüger's conclusion is based. The subject was thus left in an unsatistory and contradictory state, and, because of its bearing on our understanding the function of blood pigments in general, it seemed necessary to reinvestigate using a rigorously controlled technique.

METHOD

In my experiments large specimens (2·5–5 g.) of *Lumbricus herculeus* Savigny ommonly known as *L. terrestris*) were used. They were kept in damp soil and rkness in a thermostat at 10° C. for at least 3 days, sometimes several weeks, fore the experiments. The oxygen consumption of the worms was measured at mospheric pressure in gas mixtures of oxygen and nitrogen containing 20, 10, 5, 5 and 1 % of oxygen, i.e. at oxygen partial pressures of about 152, 76, 38, 19 and mm. mercury. In the carbon monoxide experiments carbon monoxide was added the gas mixtures in amounts (Table 1) sufficient to saturate at least 90 % of the emoglobin of the blood within half an hour. The oxygen consumption was easured in a Barcroft differential respirometer (Dixon, 1934) with modified spiration chambers. The respiration chambers were cup-shaped and had a side be with a tap. The worms lay on a disk of perforated zinc which was supported peg-like projections from the wall of the cup. Gas mixtures were passed in rough the side tap, entering the chamber below the zinc grid; the gas passed ound the worms and out through the tap at the end of the manometer. The gas ixtures were made up from cylinders of compressed gas, 2 l. at a time, with a gas rette. They were collected over water and were shaken with a little water before eing passed over the worms. Two worms at a time were used in the respirometer.

The respirometer was kept during the experiments in a water thermostat at ° C. The chambers were hooded in black velvet in order to prevent, in the carbon onoxide experiments, the dissociation of carboxyhaemoglobin by light; this rocedure also tended to standardize conditions, for the worms are strongly ffected by light.[1] At the end of the experiment, the worms, which were always vely and in good condition, were removed from the apparatus, quickly washed ee of soil, dried gently with a cloth, and weighed.

Estimations were made of the amount of carboxyhaemoglobin present in the lood of carbon monoxide-treated worms at the end of the respiration experiments,

[1] In two experiments, the worms were first exposed to darkness and then to diffuse daylight; eir rate of oxygen consumption rose by about 30 %. Davis & Slater (1928) found that the rate as doubled in bright light.

Table 1. *Mean rates of oxygen consumption, etc. at 10° C. at various oxygen and carbon monoxide pressures; P, probability that the bracketed means are of samples of the same population*

Experimental series	No. of experiments	Partial pressures of oxygen and carbon monoxide, mm. mercury		Rates of oxygen consumption c.mm. at N.T.P. per g. per hr.			Tests of significance of difference between B_1 and B_2 by analysis of covariance	
				1st hr.	2nd hr.			
		1st hr.	2nd hr.	Mean	Mean	*P*	Adjusted mean (log)	*P*
1	9 8	152 O_2 152 O_2	152 O_2 152 O_2+76 CO	A_1 45·2±4·8 A_2 43·5±4·0	B_1 38·7±4·3} B_2 29·6±3·3}	0·1	1·2698±0·0166} 1·1565±0·0177}	<0·001
2	14 12	76 O_2 76 O_2	76 O_2 76 O_2+76 CO	A_1 42·5±2·1 A_2 46·2±1·8	B_1 35·2±1·8} B_2 25·2±1·7}	<0·001	1·2650±0·0133} 1·0775±0·0144}	<0·001
3	13 10	38 O_2 38 O_2	38 O_2 38 O_2+38 CO	A_1 25·0±1·7 A_2 28·2±0·8	B_1 23·3±1·3} B_2 15·7±0·6}	<0·001	1·2916±0·0139} 1·0705±0·0158}	<0·001
4	10 6	19 O_2 19 O_2	19 O_2 19 O_2+19 CO	A_1 16·7±1·3 A_2 17·8±2·3	B_1 15·4±1·2} B_2 12·9±3·0}	0·3	1·2521±0·0158} 1·1448±0·0204}	0·002
5	3 8	8 O_2 8 O_2	8 O_2 8 O_2+19 CO	A_1 7·2±1·0 A_2 7·6±0·8	B_1 6·7±0·8} B_2 7·0±0·7}	0·8	1·1908±0·0288} 1·1980±0·0177}	0·8

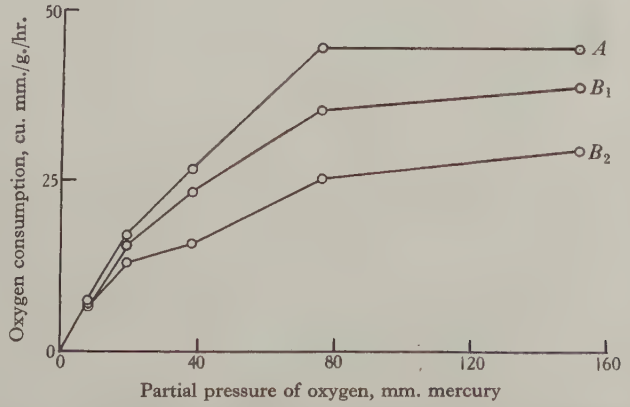

Fig. 1. Mean rate of oxygen consumption of earthworms at 10° C. at different oxygen pressures (experimental results). *A* during the first hour, in absence of carbon monoxide. B_1 during the second hour, in absence of carbon monoxide. B_2 during the second hour, in presence of carbon monoxide.

nd also of worms exposed for only half an hour to the gas mixtures containing arbon monoxide which were used in experiments, since the measurements of spiration began after half an hour's exposure. In all cases at least 90 % of the emoglobin was in the form of carboxyhaemoglobin.

The amount of carboxyhaemoglobin was estimated with a Hartridge reversion ectroscope. A calibration curve showing the relation between the spectroscope ading and the percentage of carboxyhaemoglobin in the blood had previously en made in the way described by Hartridge (1912, 1913) and Frederick (1931, 937). For this purpose blood was extracted from several worms and diluted so at, when the blood was examined in the troughs with the spectroscope, the α-band ppeared as broad as the space between the α- and β-bands. For the estimation of e amount of carbon monoxide in the blood of the experimental worms, the ordinary chnique used for human blood, which involves dilution, was avoided because lution (with air-saturated water) causes dissociation of earthworm carboxyhaemo-lobin. That the relative affinity of earthworm haemoglobin for carbon monoxide nd oxygen is less than that of human haemoglobin may be deduced from the fact at the 'span' of earthworm haemoglobin is short (Anson *et al.* 1924). The pectroscope was therefore fitted to a microscope and an undiluted drop of blood as examined with it. The blood, removed quickly with a pipette from the pseudo-earts, was put on a slide with a coverslip which could be screwed up and down. n this way the thickness of the film of blood could be adjusted so that its absorption pectrum had the same intensity as that used for calibration.

The simplest method of assessing the importance of haemoglobin in oxygen onsumption is to compare, in any one experiment, the oxygen consumption of orms, before, during, and after exposure to carbon monoxide. This was found to e impracticable, however, for two reasons. Firstly, it was found that when readings ere taken continuously for some hours, the rate of oxygen uptake was not con-tant, but declined gradually. This fall in respiratory rate with time is presumably ue to the gradual recovery of the worms from the disturbance caused by setting p the experiment, and is comparable with that found in fishes by Keys (1930). econdly, when a worm had absorbed carbon monoxide, it retained the gas for ome hours after it had been put into an atmosphere free of carbon monoxide. This nethod would therefore require that the worms should be left for many hours in he apparatus, but when this is done the worms become limp, and it is inadvisable o measure their respiration in this condition. An alternative method is to compare he mean rate of oxygen consumption of two lots of worms, one in the absence and ne in the presence of carbon monoxide. The individual variation in rate of oxygen onsumption among the worms is, however, so great that an excessively large umber of experiments would have to be made to get a reliable result. A modifica-ion of this method was therefore used which allowed the comparison of normal and arbon monoxide-treated worms in quite similar conditions, and which, as described elow, also has statistical advantages.

The worms were placed in the apparatus, and 1 l. of the required mixture of xygen and nitrogen, which had first circulated through a coil of tubing in the

thermostat, was then passed through both chambers of the respirometer, the passage of the gas taking half an hour. Five minutes after the flow of gas had stopped, the taps were closed, and manometer readings were taken every 10 min. during 1 hr. Then another litre of gas was passed through, again taking half an hour. This second lot of gas was either identical with the first (in the control experiments), or it contained carbon monoxide. After an interval of 5 min. the taps were again closed and readings were taken every 10 min. for another hour.

RESULTS

The experimental results are given in Table 1 and Fig. 1, and from them the following conclusions can be drawn. Firstly, the rate of oxygen consumption during the first hour (i.e. when no carbon monoxide is present) is the same at 152 mm. as at 76 mm., while below 76 mm. it falls sharply. This is shown clearly by the values of A, the mean rates of oxygen consumption of all worms during the first hour, which are obtained from the means of A_1 and A_2 (Table 1) weighted for the number of experiments. The values of A are, in c.mm. of oxygen per g. per hr., $44{\cdot}4 \pm 3{\cdot}0$ at 152 mm. pressure, $44{\cdot}4 \pm 1{\cdot}4$ at 76 mm., $26{\cdot}4 \pm 1{\cdot}1$ at 38 mm., $17{\cdot}1 \pm 1{\cdot}2$ at 19 mm., and $7{\cdot}5 \pm 0{\cdot}6$ at 8 mm. (Fig. 1).

Secondly, as stated above, even without carbon monoxide the mean rate of oxygen uptake is lower in the second hour (B_1) than in the first hour (A_1).

Thirdly, Table 1 and Fig. 1 show that the mean respiratory rate in the second hour in the presence of carbon monoxide (B_2) is lower than that in its absence (B_1) at all oxygen partial pressures above 8 mm. The coefficients of variation of the rates of oxygen consumption are, however, high, and while the difference between the means in the absence (B_1) and presence (B_2) of carbon monoxide, as indicated by the probability P given by the t test (Fisher, 1938), are seen from the table to be significant at 76 and 38 mm. of oxygen, at the other pressures they are not significant.

The large variation in B_1 and B_2 is partly correlated with variation in initial oxygen consumption (i.e. in first hour rates) and with variation in weight of worms (the oxygen consumption per g. per hr. shows a significant negative correlation with the weight of the worms, r being $-0{\cdot}8$). Taking advantage of this it is possible to test the significance of the difference between the means in the absence and in the presence of carbon monoxide in a more refined way, by means of the technique of analysis of covariance. I am much indebted to Prof. R.A. Fisher and to Dr W.L. Stevens for pointing this out, and for indicating the procedure necessary. By applying this technique, the effect of presence or absence of carbon monoxide in the second-hour measurements can be estimated without the obscurity produced in the original data by the sources of variation referred to above. In making the analysis, the log oxygen consumption in the second hour is taken as the dependent variate, y, and the log oxygen consumption in the first hour and log weight as independent variates, x_1 and x_2 respectively. From the regression equation $y = a + b_1 x_1 + b_2 x_2$ an adjusted mean, the mean of a, is obtained for each set of

periments, and this depends only on whether carbon monoxide is present or sent, and on oxygen pressure. These adjusted means (which are of course in the m of logarithms) are set out with their standard errors in Table 1. At each oxygen essure the difference between the adjusted means of the carbon monoxide-treated d control sets are compared with the standard error of the difference. In all es except at 8 mm. the differences prove highly significant, as indicated by the ues of P given by the t test, shown in Table 1. The elimination of extraneous riability by this technique makes it clear, therefore, that haemoglobin functions an oxygen transporter at 152, 76, 38 and 19 mm. pressure of oxygen, but not 8 mm.

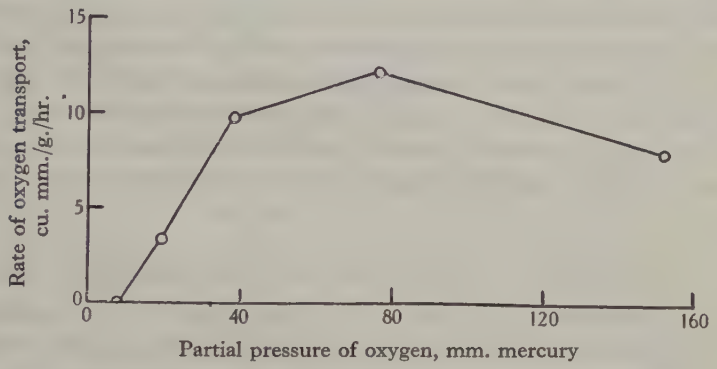

Fig. 2. Rate of oxygen transport by haemoglobin during the second hour at different oxygen pressures (10° C.).

An estimate can be made of the oxygen consumption in c.mm. per g. of worm er hr. which is mediated by haemoglobin at different oxygen pressures, and of the ercentage this represents of the total oxygen consumption. An adequate estimate nnot be made directly from the original data because of the variation in initial xygen consumption (first-hour rate) and in weight. A more reliable estimate can, owever, be obtained by adding to the adjusted values used above for each experiental set the values of b_1x_1 and b_2x_2, taking for x_1 the mean log oxygen consumption in the first hour at the given oxygen pressure, and for x_2 the mean log weight f worms in all the experiments. The difference at any oxygen pressure between the ets treated with carbon monoxide and those not treated shows the amount of xygen transported by haemoglobin at this pressure, when allowance has been ade for difference in initial oxygen consumption and weight.

The values with approximate standard errors thus obtained for the oxygen nsumption in c.mm. per g. of worm per hr. mediated by haemoglobin at the fferent oxygen pressures studied are as follows: $8{\cdot}0 \pm 1{\cdot}7$ at 152 mm., $12{\cdot}3 \pm 1{\cdot}3$ 76 mm., $9{\cdot}8 \pm 0{\cdot}9$ at 38 mm., $3{\cdot}4 \pm 0{\cdot}8$ at 19 mm. and 0 at 8 mm. (Fig. 2). Below 3 mm., therefore, the effectiveness of the haemoglobin decreases rapidly. This n be explained only by assuming that the pigment is not completely saturated at e surface of the body at 19 mm. The loading pressure (pressure at which the gment is 95 % saturated with oxygen) of earthworm haemoglobin must therefore

be higher than 19 mm. This is much higher than the figure (5 mm.) found for *Arenicola* haemoglobin by Barcroft & Barcroft (1924).

The percentage of the total oxygen consumed by the worm at a given pressure which is transported by haemoglobin can also be obtained from the estimates given above. These values and their approximate standard errors are $23 \pm 5{\cdot}0$ % at 152 mm., $35 \pm 3{\cdot}7$ % at 76 mm., $40 \pm 3{\cdot}8$ % at 38 mm., $22 \pm 5{\cdot}3$ % at 19 mm., and 0 at 8 mm. Krüger's figures are similar to these (27 % at 152 mm., 34 % at 114 mm., 36 % at 76 mm., and 31 % at 38 mm.).

Two qualifications should be made of these estimates of the respiratory significance of earthworm haemoglobin. In the first place it should be pointed out that these experiments were made on quiescent worms at 10° C., a comparatively low temperature. In conditions of higher metabolic rate the importance of haemoglobin as an oxygen transporter would presumably be greater, at least at the higher pressure of oxygen. Secondly, the amount of oxygen transported by haemoglobin as estimated by the carbon monoxide method will be a little too low, for, as is shown below, carbon monoxide increases slightly the rate of respiration of the tissues.

OXYGEN CONSUMPTION OF TISSUE SLICES

The above experiments have shown that the oxygen uptake of earthworms is lowered by the presence of carbon monoxide. Before ascribing this effect to the elimination of haemoglobin alone, it is necessary to determine whether carbon monoxide depresses also the activity of the respiratory enzymes, although the relative proportion of carbon monoxide to oxygen was low enough to make this improbable.

The oxygen consumption of slices of earthworm tissue was therefore measured in the presence and absence of carbon monoxide. The experiments were made at temperatures between 15·2 and 17·7° C. and at the highest partial pressure of oxygen (152 mm.) used in the experiments of whole worms, in order to examine the effect of carbon monoxide when the rate of oxygen consumption was high. The gas mixture containing carbon monoxide consisted of 20 % carbon monoxide, 20 % oxygen and 60 % nitrogen, and for the control experiments, a mixture of 20 % oxygen and 80 % nitrogen was used. Thus the relative proportion of carbon monoxide to oxygen was the same as that used for the experiments on the whole worms at 76, 38 and 19 mm. partial pressure of oxygen, and was twice as high as that used at 152 mm. pressure of oxygen.

The technique used was similar to that employed by Ewer & Fox (1940) for their experiments on tissue slices of *Sabella*. The oxygen consumption was measured in Barcroft differential respirometers the flasks of which were provided with side-taps so that a stream of gas could be passed over the tissues. The slices were put in the respiration flasks in 3 c.c. of frog's phosphate-Ringer solution at *p*H 7·4. This is a suitable medium for earthworm tissue, for Wu (1939) found that the activity of the earthworm gut continued for many hours when bathed in frog's Ringer solution.

Slices of earthworm tissue were prepared in the following manner. The body f the worm posterior to the clitellum was opened longitudinally and the gut con- nts washed out with frog's Ringer solution. The body wall, which is about 1 mm. ick, was cut into strips about 1 cm. long and not more than 1 mm. thick (see wer & Fox, 1940, for justification of the use of slices of this thickness).

Two respirometers were used in each experiment. The tissue in one apparatus as subjected to carbon monoxide, while that in the other was used as a control. issue from two worms was used in each experiment, and the tissue slices were oroughly mixed and then divided into two lots, one of which was put in each spirometer. By using tissue slices of the same origin in both the carbon-monoxide xperiment and the controls, the effect of individual variation was eliminated.

One litre of the gas mixture was passed through both flasks of the respirometers hile the apparatus was being shaken in the thermostat. After the gas mixture has assed for $\frac{1}{2}$ hr. the apparatus was left shaking for a further 10 min. to allow for mperature and pressure equilibration. The taps were then closed and the rate of spiration was measured. The rate of respiration was found to be constant for at ast $1\frac{1}{2}$ hr., after which it decreased slightly in some cases. At the end of the xperiment the tissue slices were filtered off in a Gooch crucible, washed, dried at 07° C. for 24 hr. and weighed. The average dry weight of tissue used in each spirometer was 83 mg. The apparatus was shaken at a rate of 120 oscillations per in. with an excursion of 5 cm. An increase in the rate of shaking did not cause an crease in the rate of movement of the fluid in the manometer. The flasks of the spirometers were covered with black velvet to exclude light.

Table 2. *Oxygen consumption of tissue slices of earthworm in* 20 % *oxygen with and without* 20 % *carbon monoxide*

Exp. no.	Temp. (° C.)	Oxygen consumption c.mm. per g. (dry)* per hr.		Rate in presence of carbon monoxide as % of rate in absence
		Without carbon monoxide	With carbon monoxide	
1	15·2	328	432	132
2	15·6	462	502	109
3	15·9	579	676	117
4	15·9	630	728	116
5	16·1	499	587	118
6	16·3	707	723	102
7	16·4	584	649	111
8	16·4	629	667	106
9	16·7	700	715	102
10	16·7	914	944	103
11	16·9	622	620	100
12	16·9	630	662	105
13	17·1	551	663	120
14	17·2	609	654	107
15	17·5	697	881	126
16	17·7	966	863	89
				Mean 110·2 ± 2·7

* 0·106 g. (dry) of worm tissue is equivalent to 1 g. (wet) of whole worm (average of measurements f eight worms).

The results of the experiments are summarized in Table 2. Since in each experiment the tissue in the two respirometers was of the same origin the rate of respiration in the presence of carbon monoxide can be compared with that of its control in the absence of carbon monoxide. In fourteen of the sixteen experiments the rate in carbon monoxide is higher than that in its absence, the average rate in carbon monoxide being 110 % of that of the controls. This difference is significant ($t = 3{\cdot}80$, $p = 0{\cdot}001$). The increase in rate of respiration may be due either to the burning of carbon monoxide by the tissues, as has been found to be the case in muscle and other tissues of vertebrates (Fenn & Cobb, 1932) or to the stimulation by carbon monoxide of oxygen consumption, as in yeast (Stannard, 1936).

From these experiments on tissue slices it can be concluded that the lowered rate of oxygen consumption found in the worms in the presence of carbon monoxide is due to the elimination of haemoglobin as an oxygen carrier, and not to a depressive effect of the gas on the respiratory enzymes of the tissues.

DISCUSSION

The results of this work, which agree with that of Krüger but not with those of other workers, show conclusively that the haemoglobin of the earthworm transports oxygen not only at low oxygen pressures, but also in air. This does not correspond with the generally accepted view as to the functioning of invertebrate respiratory pigments.

Lankester (1872) pointed out that haemoglobin is found in those invertebrates which are particularly active (e.g. the actively burrowing *Solen legumen*) or in those subject to low oxygen pressures (*Planorbis*, *Chirocephalus*, *Chironomus*, *Tubifex*). In general (see reviews by Barcroft, 1925; Redfield, 1933) it has been thought that among invertebrates haemoglobin is not concerned in transporting oxygen at ordinary oxygen pressures, when enough oxygen is carried by the blood in physical solution, but becomes effective only when the oxygen pressure of the medium is low. It is also held that the pigment may act as a reservoir for oxygen when animals are subjected to a deficiency of oxygen, as in burrowing marine forms at low tide. The evidence supporting these two views is usually, however, of a very indirect nature, and sometimes one view is held only because the other cannot be substantiated.

Leitch (1916) stated that the haemoglobins of *Planorbis* and of *Chironomus* larvae do not act as stores of oxygen, but act as oxygen transporters when the oxygen pressure of the environment is for *Planorbis* below 53 mm., and for *Chironomus* below 7 mm. Since this work is often quoted as showing that the haemoglobin of these animals is used only at low pressures, it should be pointed out that this conclusion is hardly justified. Leitch examined the foot of *Planorbis* with the spectroscope and found that the haemoglobin is always saturated with oxygen when the pressure of oxygen in the environment is above 58 mm., but is reduced when the pressure is below 55 mm., from which she concluded that the haemoglobin is not used at pressures higher than 58 mm. With this technique, in which the estimation of the amount of reduced haemoglobin depends on the fading of the

- and β-bands of oxyhaemoglobin, it would be extremely difficult to be sure when o reduced haemoglobin was present; and indeed in other experiments on the lood in vitro (that is, in easier experimental conditions) Leitch states that estima- ons of the amount of oxyhaemoglobin present were accurate only up to 70 %. he possibility that the haemoglobin was functioning by giving up a proportion of s oxygen at pressures above 58 mm. is therefore not ruled out. In the case of *hironomus* Leitch examined the whole animal and found that at 7 mm. oxygen ressure the blood was little if at all reduced, while at 5 mm. it was only 60 % turated. She concluded that the pigment is used in oxygen transport at pressures elow 6 mm., but not above 7 mm. Here again, however, the technique used is ot suitable for establishing the fact that the pigment is not used at higher pressures.

Barcroft & Barcroft (1924) found that the haemoglobin of *Arenicola* has a very igh affinity for oxygen, its unloading pressure (pressure of oxygen at which aemoglobin is 50 % saturated) being about 2 mm., and they suggested that its unction was not to carry oxygen but to act as an oxygen store when the worm was ubjected to oxygen deficiency. They found that the oxygen content of the blood as enough to last the worm about an hour—this period being less than, but of he same order as, the period during which the worms are closed in their tubes at w water. Borden (1931) confirmed this. She also found that in *Planorbis* the lood would hold enough oxygen to last the animal 25 min.; she considered that he oxygenated pigments were not unimportant as reserves of oxygen. From the w pressure at which the pigments dissociate (*Planorbis* 1–10 mm. oxygen, Bar- roft, 1928; *Arenicola* 1–3 mm., Barcroft & Barcroft, 1924), she argued that the aemoglobin acts as an oxygen carrier at low pressures of oxygen, but this was not emonstrated experimentally.

Redfield & Florkin (1931) found that the unloading pressure of the haemoglobin of the gephyrean worm *Urechis* is 12 mm. They decided that the oxygen bound to aemoglobin is not utilized under ordinary conditions. This conclusion is difficult o reconcile with their finding that the haemoglobin is not completely saturated in vivo, though it is saturated when in equilibrium with air. They concluded that aemoglobin acts as an oxygen transporter when the oxygen content of the worm's burrow is reduced during low tide and as an oxygen store when respiratory move- ments are suspended.

The possibility that the pigments serve a respiratory function at atmospheric pressures of oxygen is therefore not excluded by the results of any of this work. In the case of *Chironomus* both indirect and direct methods have been used to deter- mine the oxygen pressure at which haemoglobin became effective as an oxygen carrier. It is significant that the indirect method (Leitch) gave a much lower result than the direct (Harnisch, Ewer, see below).

Evidence of a direct nature as to the respiratory function of blood pigments is obtained by comparing the oxygen uptake of normal animals with those whose haemoglobin is rendered functionless as an oxygen transporter by saturation with carbon monoxide. The work of Jordan & Schwarz (1920), Dolk & van der Paauw (1929) and Thomas (1935) on the earthworm has been discussed above; the results

indicate that the haemoglobin does not carry oxygen at atmospheric partial pressures of oxygen. Harnisch (1936) found the same in *Tubifex*, but his work is open to criticism and his results do not agree with those of Dausend (1931). Harnisch also worked on *Chironomus* larvae and found that their oxygen consumption is not affected by carbon monoxide in air-saturated water but is lowered at oxygen pressures of 84 mm. and below. This work also is open to serious criticism, but Mrs R. F. Ewer (1941) in this Department, using better methods, has obtained a similar result.

Other work of this direct nature has shown that invertebrate respiratory pigments function as oxygen transporters even at atmospheric pressures of oxygen. Jürgens (1935) claimed that this was so in *Nereis*, but his experimental evidence is weak, for he worked with narcotized animals subjected to pure carbon monoxide. Dausend (1931) found that in *Tubifex* the oxygen uptake of worms treated with carbon monoxide is lower than that of normal worms even in air-saturated water. Ewer & Fox (1940) found the same in *Sabella*, whose blood contains chlorocruorin. Krüger (1938), as discussed above, found, as I did, that the oxygen consumption of worms treated with carbon monoxide is lower than that of normal worms even at atmospheric partial pressure of oxygen.

In the case of *Sabella* (Ewer & Fox, 1940), the fact that chlorocruorin functions at air pressure was to be anticipated from our knowledge of its relatively high loading pressure (Fox, 1932). The prevalent opinion that haemoglobin in the invertebrates cannot be effective in oxygen transport at anything but very low oxygen pressures may be attributed to the low loading and unloading pressures which have been found for the pigments (see Redfield, 1933). It should be pointed out, however, that for a pigment to be effective it must be loaded with oxygen at the respiratory surface and unloaded at the respiring surface; whatever its loading pressure, the pigment will act as an oxygen transporter if the respiring tissues are at an oxygen pressure lower than the loading pressure, so that the oxygenated pigment becomes, at least partially, unloaded. That the oxygen pressures inside the body of invertebrates may be quite low is shown by the work of Adler (1918), who found that the coelomic fluid of an earthworm in air had an oxygen pressure of 14 mm. The most efficient pigment for an animal which is subjected to oxygen deficiency of the environment will be one whose loading pressure is low, so that, unlike a pigment with a high loading pressure, it will be loaded at the respiratory surface even when the oxygen pressure of the medium is low.

In conclusion is may be said that, except in *Chironomus*, there is no firm evidence that invertebrate haemoglobin does not transport oxygen at air partial pressure of oxygen. Further investigations of the oxygen consumption of animals treated with carbon monoxide may show that their respiratory pigments are of value not only at low oxygen pressures but also at atmospheric partial pressure, as in *Tubifex*, *Sabella* and *Lumbricus*.

SUMMARY

The oxygen consumption of earthworms (*Lumbricus herculeus* Savigny) has been measured at 10° C., in the dark, in atmospheres containing 20, 10, 5, 2·5 and 1 % of oxygen (i.e. at partial pressures of oxygen of about 152, 76, 38, 19 and 8 mm.

ercury), with and without the addition of enough carbon monoxide to saturate ne haemoglobin of the blood.

In the absence of carbon monoxide the rate of oxygen consumption was gnificantly the same at 152 and 76 mm.; below 76 mm. it fell sharply.

The rate of oxygen consumption of carbon monoxide-treated worms was gnificantly lower than that of normal worms at oxygen pressures of 152, 76, 38 nd 19 mm. but not at 8 mm.

The respiration of slices of earthworm has been measured in atmospheres ontaining 20 % of oxygen, and 20 % of oxygen together with 20 % of carbon onoxide. The rate of respiration in the presence of carbon monoxide was 110 % f that in its absence. It is concluded that the lowering of the rate of respiration f whole worms caused by carbon monoxide was not due to inhibition of respiratory nzymes, but to its effect on haemoglobin. Haemoglobin therefore transports xygen at atmospheric as well as at lower partial pressures of oxygen.

Less oxygen was carried by haemoglobin at 19 mm. than at 38 mm. It is educed that the loading pressure of earthworm haemoglobin is higher than 19 mm.

The haemoglobin of the blood was responsible for supplying about 23 % of he respired oxygen when the oxygen pressure was at 152 mm., 35 % at 76 mm., 0 % at 38 mm., and 22 % at 19 mm. of oxygen.

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THE BIOLOGY AND BEHAVIOUR OF *PTINUS TECTUS* BOIE. (COLEOPTERA, PTINIDAE), A PEST OF STORED PRODUCTS

II. THE AMOUNT OF LOCOMOTORY ACTIVITY IN RELATION TO EXPERIMENTAL AND TO PREVIOUS TEMPERATURES

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(With Six Text-figures)

I. INTRODUCTION

It has long been known that temperature has a great effect on the speed of biological processes. For this reason, when the speed of such a process is being measured, the experimental temperature has to be controlled. It has only recently been realized, however, that the temperature at which the biological material has been kept before the experiment may also be important, even in cases when damaging temperatures are not in question. For example, Thompson (1937) recorded the rate of heart beat of embryos of certain grasshoppers; for late embryos of *Melanoplus femur-rubrum* reared and tested at 20° C. this rate was considerably higher than for similar embryos tested at the same temperature but reared at 35° C. In fact, the rate for embryos reared and tested at 20° C. was about the same as that for embryos reared at 35° C. and tested at 30° C. Mellanby (1940) found that the rates of heart beat of the crested newt (*Triton cristatus*) were much higher at both 0 and 10° C. if the animals had been kept at 10° C. merely for the previous day than if they had been kept at 30° C. for the same time. Again, Hopkins (1937) found that the rate of locomotion of an amoeba, *Flabellula mira*, depended on temperature in the usual way only if some hours had been allowed for temperature acclimatization; during that period, the rate fluctuated widely before becoming steady. On the other hand, Mellanby (1939) concluded that the speed of walking of bedbugs (*Cimex lectularius*) depended only on the experimental temperature and not at all on the previous temperature, provided the previous temperature had not been low enough to cause chill coma nor high enough to be damaging, and provided the insects were allowed half a minute to acclimatize to the new temperature.

There is some other information about the effects of previous temperatures on processes such as these, but it is scanty; our work on the subject was to have been a comprehensive study of *Ptinus tectus*, but it was interrupted at an early stage by injuries sustained by one of us (H.S.H.) during an air raid. The incomplete results

locomotory activity are presented now because they include new and useful information and because there is no immediate prospect of extending them.

Locomotory activity is important in the life of this beetle not only because it is partly responsible for spreading the species to neighbouring places, but also because the effectiveness of certain lethal agents depends on how much the insects are moving about.

In order to find the total amount of movement in a given time, it is necessary to know two things: how much of the time is spent in movement, and the speed of the movement while it is taking place. An estimate of the intensity of locomotory activity could, of course, be made by means of a suitable combination of measurements of oxygen consumption; our work, however, deals only with the amount of time spent on locomotion. It will be seen that this does depend on the previous temperature as well as on the temperature during observation.

Up to the present, three kinds of observation have been made on the effects of temperature on the locomotory activity of invertebrates. First, Crozier (1924) measured the rate of walking of a millipede, *Parajulus pennsylvanicus*, as a function of temperature, the object being to find the 'critical thermal increment'. Second, Chapman *et al.* (1926) raised the temperature of a vessel containing various insects at a rate of 21° C. per hour, and recorded qualitatively the kind of activity—first movement of a limb, first crawling, first sign of heat paralysis, etc.—corresponding to each temperature. Third, Nicholson (1934) estimated the proportion moving in a batch of blowflies under given temperature conditions; since this is essentially the measurement we have adopted, it will be dealt with more fully.

In assessing the activity of *Ptinus*, the kind of activity was practically ignored. Locomotion was recorded separately from mere movements of the legs or antennae which did not result in the insect changing its location ('virtual inactivity'), but no special attention was paid to the speed of locomotion. *Ptinus tectus* does not fly. When separate curves were drawn, showing locomotory activity alone and activity plus 'virtual inactivity', the latter were somewhat the higher, but there was no essential difference in shape. The general conclusions to be reached are not affected by discussing or including the records of 'virtual inactivity', so they are omitted for simplicity. We are concerned simply with the number of animals in locomotion under given conditions.

The observations of activity were made by the 'cross-section method' (Bentley *et al.* 1941). The aim is thus to count the animals which are moving at one instant; if an individual observation were to take an appreciable time, then the number of animals counted as moving would increase as the duration of this time increased, for some individuals would start to walk during the count. By this cross-section method, one might, for example, find that 60 % of the animals were moving at an instant; with a reasonably constant level of activity over a period, it can be inferred from this that *on the average* each animal walked about for 60 % of the period in question. If individual animals were to be considered, it would of course be found that the time spent in activity varied from one individual to another.

Some of the factors, other than temperature, which affect the activity of *Ptinus*

were easily eliminated: thus the normal diurnal rhythm of activity was abolished by keeping the insects long enough at constant temperature and in unvarying light (Bentley *et al.* 1941); observations were usually not started for several hours after the animals had been placed in the observation chamber, so that the activity due to the mechanical stimulation of handling had had time to subside; as far as possible, the light intensity in the observations was the same as for the cultures. With the aid of these precautions, reasonably self-consistent results were obtained, but very smooth curves are not to be expected, because the activity of *Ptinus* appears to be sensitive to many conditions.

In considering the effects of temperature both before and during the observation period, there appears to be only one set of conditions in which no arbitrary choice of temperature or time relations has to be made; that is, the set of conditions in which a number of batches of animals are bred at different temperatures and the activity of each batch is assessed at its temperature of breeding. Such a procedure allows only a limited comparison to be made between different batches. If animals are to be tested at a temperature other than the temperature of breeding, then the speed at which their temperature is changed and the time they are left at the new temperature before testing have to be chosen arbitrarily. Had sufficient data been accumulated, it might have been possible to reduce the arbitrariness of the choice of conditions. The conditions chosen are mentioned under the appropriate headings.

II. MATERIAL AND METHODS

The experimental animals were normally bred and kept at 25° C., except when the effects of a different culture temperature were to be investigated. They were bred in 2½ l. jars (Breffitts) containing about 600 g. of wholemeal flour and 30 g. of dried yeast; the animals could drink water from a tube filled with wet cotton wool; some pads of dry cotton wool lay on the flour, and animals for experiments were usually picked off these pads. The light was on constantly, day and night, and its intensity was about 25 m.c. around the cultures. The animals used were of mixed and unknown ages: during the observations of activity they were neither fed nor allowed to drink; none of them was used more than once.

The observations were carried out with the animals in glass dishes immersed in water baths. The type of dish was cylindrical, 16 cm. in diameter and 8·5 cm. high, with the rim ground flat to make a good joint with a plate glass lid. This lid was held on by a pair of joiner's clamps, which also served to sink the dish, and the joint was made air tight with vaseline and, at temperatures over 30° C., an outside layer of paraffin wax. There were two holes in the lid, one for a thermometer and the other carrying a piece of 6 mm. glass tubing through which the animals were dropped into the dish. This tubing was not stoppered, but a piece of capillary tubing was fixed to the open end with rubber tubing, thus preventing gross exchanges of air between the dish and the outside but allowing pressure adjustments. The beetles could walk about freely on a perforated zinc platform, but were prevented from getting around the edges and below the platform by a ring of electric flex, freed

from its insulating cloth and rubber, squeezed into the small gaps between the platform and the glass wall. Below the platform there were glass pots of potassium hydroxide or sulphuric acid solution of appropriate concentration to control the humidity. Observations were almost always made concurrently at three different humidities (approx. 34, 60 and 95 % R.H.); the effect of humidity on activity was small and uncertain compared with the effects of temperature and it will not be dealt with here. Five animals were used in each dish in each experiment.

One of the two water baths used for temperature control was a refrigerated thermostat designed and constructed by Mr R. J. Whitney. There was artificial illumination of about 25 m.c. around the dishes, the tank being completely enclosed in wood. This tank was used for experiments at constant temperatures between 1·5 and 15° C. The other water bath could be used as a thermostat with the normal arrangement of a 400 W. heater, mercury-toluene regulator and Sunvic vacuum switch. For experiments between room temperature and tap-water temperature, cooling was carried out with tap water passing through metal tubing. This tank was surrounded by light-proof curtains and, once more, the light intensity was about 25 m.c. This second tank could be heated rapidly with bunsen burners and cooled with ice or a stream of water.

III. OBSERVATIONS AT CONSTANT TEMPERATURE

(*a*) *Animals bred and kept at* 25° C.

The first observations were made on the activity of animals bred and kept at 25° C.; they were transferred to the experimental dishes, which had already been allowed half an hour to equilibrate in the thermostat, and then left at the constant temperature of the particular experiment for over 24 hr. The first reading was taken after 23 hr., and nine further readings were made at 10 min. intervals. For each of the three dishes with five animals in each, the number of records of activity from one experiment was thus fifty. Each point on Fig. 1 shows what percentage of these fifty records were records of animals moving from place to place. Thus two series of experiments each with three dishes show that at 10° C. averages of between 10 and 18 % of the animals were walking about at any instant.

In Fig. 1 the seventy-one points represent observations on that number of different batches of five animals. Generally speaking, this graph shows a surprising self-consistency. The only notable inconsistency was at 20° C.; the three batches in the second series at this temperature were all rather inactive, and no explanation has been found for this.

In Fig. 2 the six sets of observations of Fig. 1 are shown combined in the curve marked 25° C.

(*b*) *Animals previously kept at temperatures other than* 25° C.

A preliminary series of experiments was done with animals which had been kept cooler than 25° C. A culture of young adults (less than a month old) was simply immersed in a tank which was kept cool with tap water. Between the beginning of

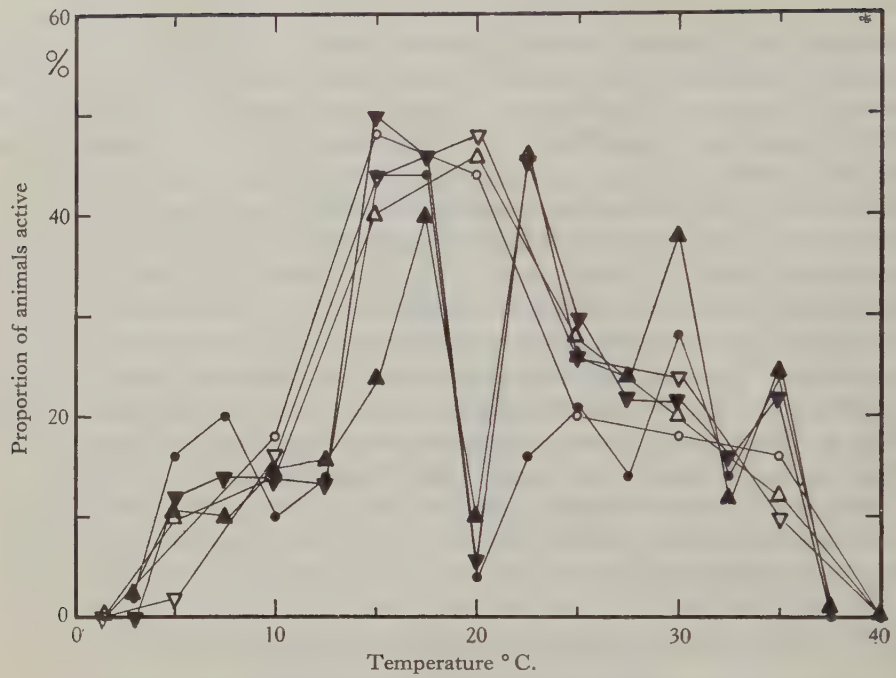

Fig. 1. Proportion of adult *Ptinus tectus* which were walking about after a day at constant temperature. Each point represents ten observations on five animals.

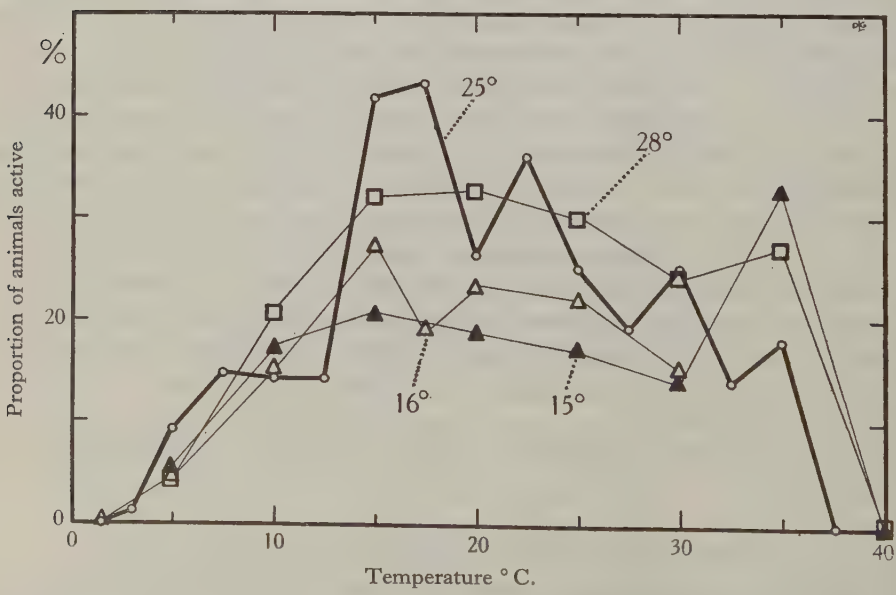

Fig. 2. Activity of *Ptinus* after a day at constant temperature. The animals were bred (25° C.) or kept, for at least three weeks before the experiment, at the temperature indicated near each curve and then kept for a day at the constant temperature of observation.

he experiments in May and the end in July, the temperature rose from 13·5 to 8° C. The observations began after 3 weeks and the culture temperature was bout 16° C. when most of the experiments were done. In other respects the xperiments were like the previous ones.

The results are shown in the curve marked 16° in Fig. 2, and they were generally imilar to those for a previous temperature of 25° C., but the activity recorded was ess. Further experiments were therefore done with carefully controlled previous emperatures of 15 and 28° C. It was not then known that 28° C. is too high a emperature for the development of *Ptinus* (Ewer & Ewer, 1941), but it does not eem to have been too high for the purpose of these experiments.

These two sets of observations were like each other, but different from the two previous sets in that the cultures had to be kept in constant darkness. At each temperature and humidity two batches of five animals were studied simultaneously, one from 15 and one from 28° C. Since all the animals had originally been taken rom one culture, the two sets of experiments are comparable.

It will be seen from Fig. 2 that when observed at any temperature between 10 and 30° C. the activity of animals previously kept at 28° C. was always higher than that of animals previously kept at 15° C., the increase being about 60 % on the average.

Leaving aside for the moment the differences between the four curves of Fig. 2 and considering only their general shape, it will be seen that they are mutually confirmatory. They have been combined in the lowest curve of Fig. 4; in this case, a number of the set of experiments for a previous temperature of 25° C. (e.g. at 7·5, 12·5, 17·5° C., etc.) have been omitted, so as to avoid unduly weighting the curve with this set.

Up to an experimental temperature of 15° C. the activity after a day at constant temperature is higher at each successively higher temperature. This is in line with well-known physiological effects of temperature. But between 15 and 25° C. the activity is relatively constant. It seems very unlikely that activity in this broad temperature region reaches a maximum because of harmful effects of temperature. For example, Deal (1939) has shown that *Ptinus* has a preferred temperature zone around 23° C. and this has been confirmed by Dr Gunn and Miss Walshe in this laboratory. Ewer & Ewer (1941) have shown that the life history of *Ptinus* is shortest at 23–25° C. and they did not find any harmful effects of temperature on adults at 25° C. or below. We have had generation after generation of *Ptinus* in cultures kept at 25° C. Indeed, Fig. 2 itself shows that after a day at 35° C. *Ptinus* walks about to much the same extent as it does after a day at 15° C., and this activity was not notably abnormal in character. We therefore consider that the shape of the curves between 15 and 25° C. in Fig. 2 represents the relation between temperature—previously constant for a day—and the frequency of normal non-pathological locomotory activity. This relation is quite unlike that approximately expressed by the Q_{10} rule. Indeed, if one does not attach much value to the somewhat higher average activity at 15° C., one may say that the frequency of locomotory activity of *Ptinus* (*not* the velocity of locomotion) does not vary much between 15 and 35° C., provided the temperature has been constant for a day.

IV. OBSERVATIONS WITH CHANGING TEMPERATURE

In some of the experiments just described, observations were made incidentally during the hour after transfer of the insects to the experimental dishes. Instead of the average activity being about 30 % as it was after a day, it was usually higher than 60 %, especially between 12·5 and 37·5° C. Part of this high activity was doubtless due to the recent handling which the animals had had, so that in investigating the other factor—recent change of temperature—the animals were placed ready for observation a day before observation started.

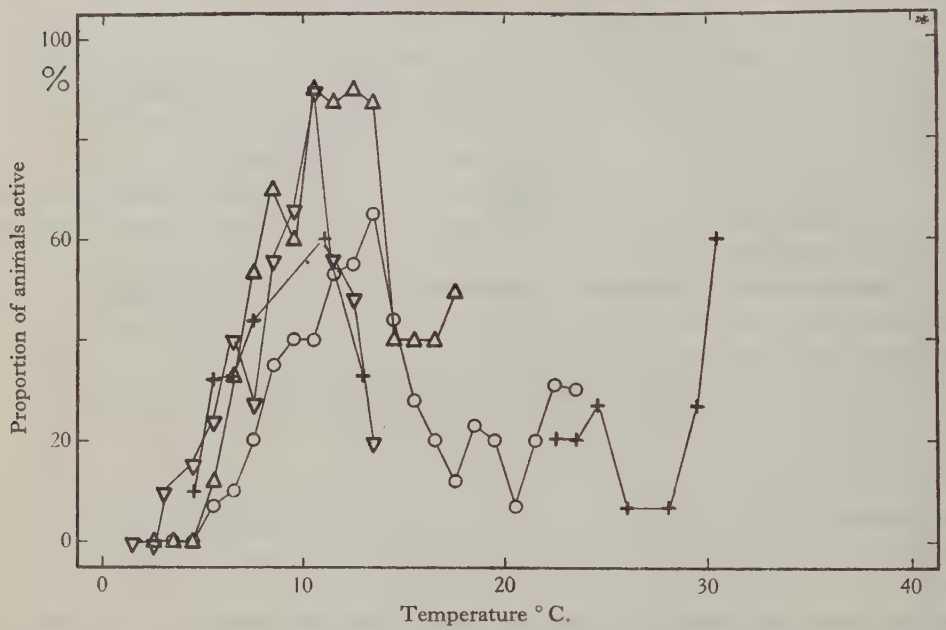

Fig. 3. Activity of *Ptinus* kept overnight at 2° C., when the temperature is then raised at between 3 and 7·5° C. per hour. Note that activity is shown at half the scale of Figs. 1 and 2. Each series of symbols represents the combined results from three or two (one series) batches of five animals each.

The apparatus was essentially as before, but air temperature inside the experimental dish was estimated with a thermocouple, because a mercury thermometer follows air temperatures too sluggishly. The wires of the couple were rather thick, so that the readings were affected by the temperature outside the dish, and consequently the rates of temperature change given below do not pretend to precision.

The observation chambers were prepared and, with the animals inside them, were left at about 2° C. overnight. Each chamber was then moved to the water bath for observation and the water temperature was raised at between 3 and 7·5° C. per hour. The results of four experiments with eleven different batches of animals are shown in Fig. 3. Activity rose to a maximum at between 10 and 15° C. and then fell. In a solitary experiment (three batches of animals) there was a second rise at 30° C. Thus at about 10° C. activity amounted to 40–90 %, instead of 20 % or

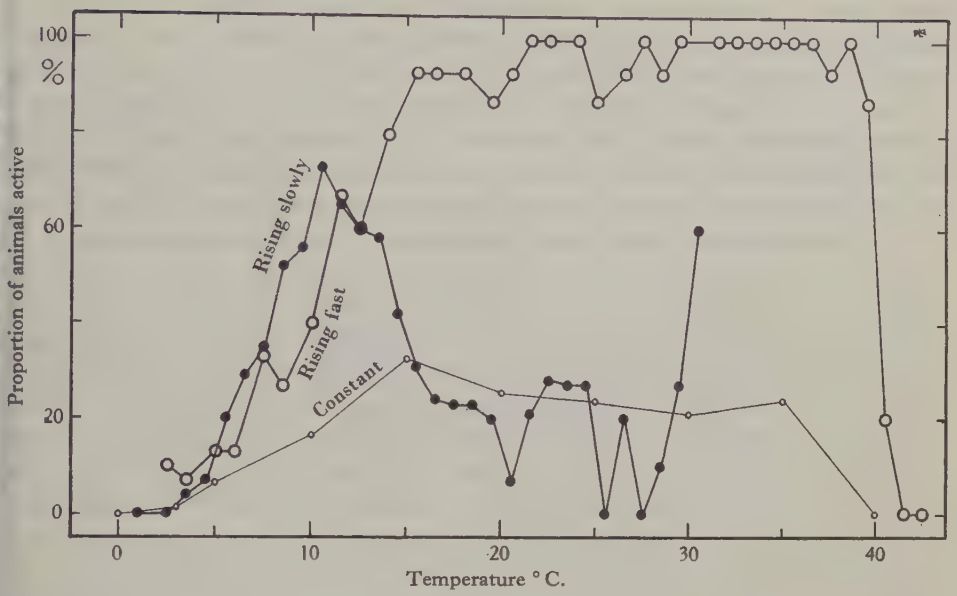

Fig. 4. Activity of *Ptinus* when the temperature is raised fast, at about 14° C. per hour. There are also shown, for comparison, the combined results of Fig. 2 (constant temperature) and Fig. 3 (slowly rising temperature).

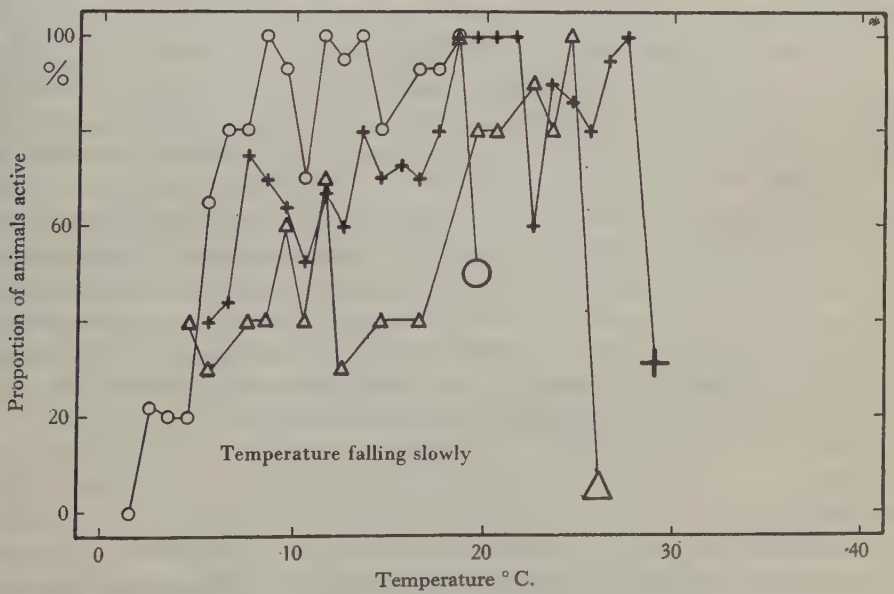

Fig. 5. Activity of *Ptinus* with slowly falling temperature (4–7·5° C. per hour). The large symbols indicate the initial temperature, which had previously been constant overnight. Each series of points represents the combined results of three or two (one series) batches of five animals.

below as in the experiments at constant temperature. Between 20 and 30° C., however, activity was much the same whether the temperature was constant or rising at the fairly slow rate of about 1° C. in 12 min.

When a faster rate of temperature rise was used—about 14° C. per hour—the resulting activity reached a high level at 15° C. and remained near 100 % until lethal effects began at about 40° C. Fig. 4 shows how different the activity levels were with the slower and faster rates of temperature rise.

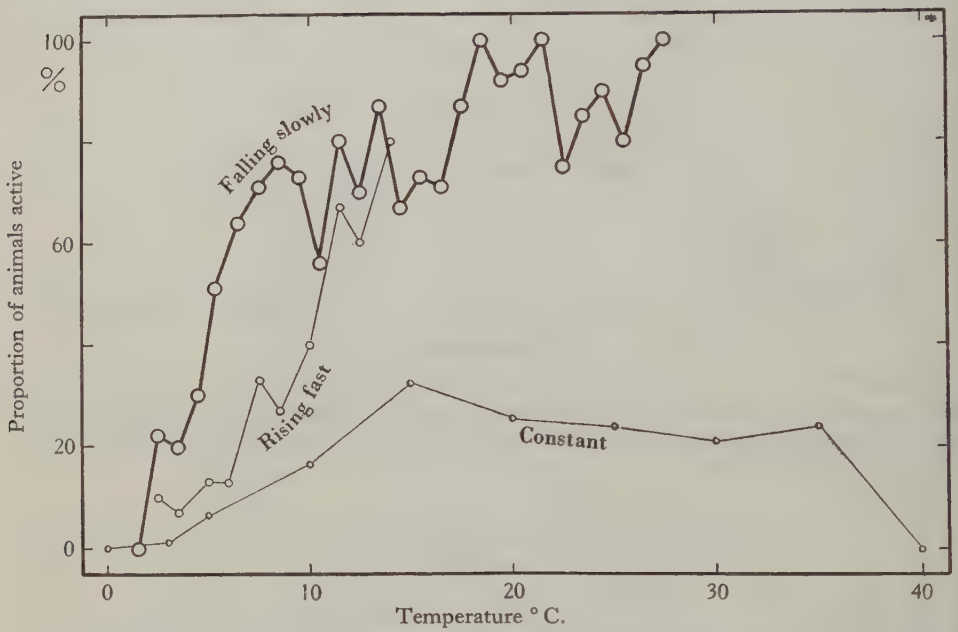

Fig. 6. Activity of *Ptinus* with slowly falling temperature, showing the combined results of Fig. 5. There are also shown, for comparison, the combined results of Fig. 2 (constant temperature) and, up to 14° C., the results from Fig. 4 with rapidly rising temperature.

Three experiments were carried out with the temperature falling at between 4 and 7·5° C. per hour, using eight batches of five animals each. The initial temperatures, at which the animals were kept for the day preceding observation, were 19, 27 and 29° C. The results are shown separately in Fig. 5 and combined in Fig. 6. It is perhaps surprising to find that when the temperature is falling slowly, the animals are usually as active at a given temperature as when it is rising fast; below 10° C. they seem to be more active on cooling (Fig. 6).

V. DISCUSSION

The results given above, incomplete though they are, are sufficient to indicate the varied ways in which the frequency of locomotion of *Ptinus* depends on temperature—culture temperature, the temperature for the previous day, and the temperature, falling or rising fast or slowly, in the previous few minutes. At this stage the results are suggestive, but analysis awaits more complete observations.

It is well known that the rate of frequency of the most varied biological processes, such as oxygen consumption, mitosis, growth and even walking, increases with temperature in a way which can be approximately expressed in the Q_{10} rule or, what comes to much the same thing, the Arrhenius formula (Bělehrádek, 1935; Heilbrunn, 1938). The value of Q_{10} is often about 2 or 3, as long as the temperature region considered is not too close to or above the optimum. Examination of our curves shows no resemblance whatever to the usual type of Q_{10} curve, unless the optimum is considered to be at or below 15° C., for above this temperature activity rises little in any of the temperature conditions we have dealt with. It would be reducing the already difficult conception of optimum to the absurd to regard 15° C. as optimal in any way for *Ptinus*; this temperature lies at or below the middle of the temperature range in which *Ptinus* can develop and carry on its normal activities (Ewer & Ewer, 1941). It is not particularly surprising that the frequency of locomotion of *Ptinus* does not follow the Q_{10} rule; what is surprising, even with selected data, is that the velocity of walking of certain animals does roughly follow this rule.

In Nicholson's (1934) experiments on *Lucilia cuprina*, activity after 12 hr. at constant temperature was highest at 30° C., which is about the preferred temperature, and fell away rapidly above and below that temperature; in temperature rising at about 7° C. per hour, activity was actually less at medium temperatures and there appeared to be a stimulating effect of changing temperature only outside the preferred zone, below 15 and above 37° C. This corresponds roughly with our results for *slowly* rising temperature (3–7·5° C.), as far as they go. But with slowly *falling* temperature the activity of *Ptinus* is much greater, and so it is with quickly rising temperature (14° C. per hour). These results demonstrate an effect of temperature change as distinct from temperature level. Such an effect has already been shown by Kennedy (1939) in experiments on the locust, *Schistocerca gregaria*, in the Sudan. A sudden change of temperature eventually resulted in the locusts reaching a new activity level corresponding to the new temperature, but the immediate result of the change was a rise above the appropriate constant level. Indeed, when the temperature fell there was an absolute rise in activity which was more prolonged than the temporary rise due to a rise in temperature. In this work of Kennedy's, the change of temperature was very rapid, being initially 1° C. in 10–15 sec.

Our work reveals a weakness of the observations made by a number of authors on the effect of rising temperature on insect activity. They raised the temperature at rates of about 7° (Nicholson, 1934), 10° (Bodenheimer *et al.* 1929), 11° (Hussein, 1937), 20° (Nieschulz, 1933, 1935), and 21° C. (Chapman *et al.* 1926) per hour. But the temperature at which most of the animals are first active in our experiments is about 10° C. when the temperature is raised at 3–7·5° C. per hour and about 15° C. when it is raised at about 14° C. per hour. In fact, over the range of rates which have been used by the various authors, the rate itself is likely to be as important as the temperature in determining the point of first maximum activity; the other standard points may be affected in a similar way.

Nicholson's work (1934) on activity in blowflies seemed to indicate a relation

between activity, as measured by the method we have adopted from him, and temperature preference (Fraenkel & Gunn, 1940, pp. 206–11). In the temperature gradient apparatus in use in this laboratory (Gunn, 1934, 1935), however, *Ptinus* can walk along the gradient at a rate equivalent to 10° C. per *minute* or 600° C. per hour! It would therefore be unwise to attempt to draw from our experiments any conclusion whatever about the mechanism of preferred temperature in this particular gradient.

VI. SUMMARY

The amount of walking about shown by adult *Ptinus tectus* is not a simple function of temperature. It depends on culture temperature, on whether the temperature is constant or changing and, with changing temperatures, on the speed and direction of change. Thus, at 20° C., about 25 % of animals walk about if the temperature is constant or rising slowly, while about 90 % are active if it is rising fast or falling slowly. None of the temperature-activity curves bear any resemblance to the usual kind of Q_{10} curve.

For experiments in which the temperature has been constant for a day the temperature-activity curve of *Ptinus* rises from 3 to 15° C., then is steady or falling somewhat to 30 or 35° C., and then falls steeply to 40° C. The activity level depends on the temperature at which the animals have been kept during the previous weeks. When the temperature is raised slowly (3–7·5° C. per hour), activity rises steeply up to about 10° C., then falls to the level appropriate to constant temperatures and, at about 30° C., rises steeply again. When the temperature is raised more quickly (14° C. per hour) the activity rise is at first similar to that for slowly rising temperature, but it continues until 90–100 % activity is reached at about 15° C.; activity remains at this high level until damaging changes occur at about 40° C. When the temperature is slowly lowered, the activity curve is similar to that for quickly rising temperature. There is thus a stimulating effect of change of temperature—whether rising or falling—the magnitude of which depends on the speed and direction of the change.

The results obtained have a considerable bearing on the testing and use of insecticides whose effectiveness depends on the activity of the insects.

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THE BIOLOGY AND BEHAVIOUR OF *PTINUS TECTUS* BOIE. (COLEOPTERA, PTINIDAE), A PEST OF STORED PRODUCTS

III. THE EFFECT OF TEMPERATURE AND HUMIDITY ON OVIPOSITION, FEEDING AND DURATION OF LIFE CYCLE

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(With Four Text-figures)

I. INTRODUCTION

An account of the first records and subsequent spread in this country of the spider-beetle *Ptinus tectus* Boie. has been given by Bentley, Gunn & Ewer (1941). Hinton (1941) gave a list of the foodstuffs on which *Ptinus* has been found, and a summary of earlier references to the animal. Previous work on the biology of *Ptinus* has been carried out under more or less uncontrolled conditions, and provides only very approximate values for the duration of the various developmental stages. König (1936) studied the duration of embryonic development at various temperatures but without control of humidity. Fahmy (1931) gave values for the duration of larval, pupal and pre-emergence stages under uncontrolled conditions, while more recently Hickin (1940) has made a study which was mainly concerned with the effect of different foodstuffs on the durations of stages and rates of reproduction at certain temperatures and humidities.

The results given below deal mainly with the duration of the different stages under controlled conditions. They are incomplete, and in many cases only preliminary in nature; but they are put on record because the work has had to be abandoned.

II. MATERIAL AND METHODS

Specimens of *P. tectus* were supplied by the Biological Field Station of the Imperial College of Science at Slough, and by Dr N. E. Hickin. For breeding purposes the animals were kept in shallow dishes (1 lb. tongue jars) containing 1·5–2·5 cm. of wholemeal flour mixed with some dried yeast. An excavated glass block filled with cotton wool, which was kept moist, provided water for the animals to drink, while a hole in the glass lid allowed evaporation and prevented the culture from going mouldy. The adults were removed fortnightly and placed in another dish with fresh food. The eggs laid during the fortnight developed into a culture of the

xt generation, and so on. In this way, it was easy to maintain a constant supply animals of roughly known age. This method of culture was found to be much ore satisfactory than that of keeping adults for long periods in large (Breffit) tures with a layer of food 10 cm. deep or more. Not only is the approximate e of the animals known, but animals from shallow cultures appeared to be in tter condition than those from deep cultures. It is possible to assess the condition the animals by dissection. Adult animals are considered to be in good condition hey contain much fat, the gut is full of food, the testes of the males contain active erm and the ovaries of the females (except very young ones) contain ripe eggs. batch of adults was kept at room temperature (10–20° C.) in such a shallow cul-re, the flour being changed fortnightly. Samples were dissected fortnightly for months; during the whole of this time the animals remained healthy. Dissections animals from deep cultures, on the other hand, frequently showed little or no , little food in the gut, and degeneration of the gonads in both sexes.

Holdaway (1932) has shown that 'conditioning' of the flour by *Tribolium* adults s a detrimental effect on the development of subsequent populations. It may be at in *Ptinus* we are dealing with a similar phenomenon, and the shallow-culture ethod has the advantage of removing the adults from the food material every rtnight; the shallow cultures may also permit loss by diffusion of any volatile xic products, which would accumulate more easily in the deep cultures. In any se it would appear that where animals in good condition and of comparable age e required for experiments the shallow-culture method is better than the deep-lture method.

Temperature regulation was difficult, as power was occasionally cut off owing enemy action. Temperatures of 20° C. and over were maintained in electrically ated ovens controlled by Sunvic regulators. These fluctuated by not more than 0·5° C., while the average temperature was very steady. Experiments at 25 and ;° C. were also carried out in a constant-temperature room, where the fluctuation as again within ±0·5° C. Experiments between 5 and 15° C. were carried out in small Hearson oven, to which ice had to be added at frequent intervals; in this e variation was within ±0·5° C. Some experiments at 15° C. were done in a rger refrigerated unit constructed by Dr Gunn,[1] but this apparatus was then still ndergoing modification and test, and the temperature varied by ±1° C.

Humidity was controlled during the experiments by solutions of KOH as escribed by Buxton & Mellanby (1934). The relative humidities in equilibrium ith these solutions were checked by means of Edney hygrometers against solutions H_2SO_4 made up according to Landolt-Börnstein (1905). It was found that the OH solutions only approximated to the values calculated from Paranjpe's (1918) bles, and the values obtained from the calibration against sulphuric acid were sed throughout.

The food mixture used in all experiments was a mixture of 75 % National traight Run English biscuit-making flour, 20 % light white soluble casein and 5 %

[1] This special unit was paid for by a grant made to Dr Gunn by the Government Grant Committee of the Royal Society.

dried brewers' yeast. It is known that an inadequate diet lengthens the time taken for the completion of the larval stage of *Ptinus* (Hickin, 1940); the same is true of *Tribolium* (Sweetman & Palmer, 1928; Good, 1933; Fraenkel & Blewett, 1941). The diet chosen was considered to be optimal, and the durations of development found ought therefore to be minimal.

The food mixture was first adjusted roughly to the desired humidity as follows, using an apparatus made by Dr Gunn. The mixture is placed in a tin of square cross-section which can be rotated mechanically on its long axis. The square cross-section prevents slipping and ensures mixing. If it is desired to raise the humidity of the flour, a corked Soxhlet extraction thimble filled with damp cotton wool is added and rotation is continued until the desired humidity is approximately attained. If it is desired to lower the humidity, the thimble is filled with granular calcium chloride. In all cases the humidity was finally accurately adjusted by spreading the food out and allowing it to equilibrate with a standard solution of KOH in a sealed glass dish.

All the food material was sieved (100 mesh) before adjusting the humidity, and eggs were collected from the food by sieving through a 60 mesh wire sieve. Eggs thus collected were allowed to develop in small excavated blocks at the desired humidities. Sieving the food to remove eggs tends to alter its humidity, and newly adjusted food was therefore used after each sieving. *Ptinus* will seldom oviposit in empty dishes, but König (1936) has shown that, in the absence of food, eggs are laid readily in wool. Double thickness knitting wool is suitable for this purpose. Teasing out the wool frees the eggs, which can thus be obtained with a minimum of handling and completely free from the food which normally adheres to the sticky egg shell. It is desirable to use eggs collected by this method for experiments at high humidities, as the presence of food favours the growth of moulds.

The remaining stages were studied in small (0·7 cm.) specimen tubes. One animal was placed on food in each tube. The majority of larvae build their cocoons up against the glass wall of the tube and pupation can thus be observed. In adult *Ptinus* the sexes show no external differences, but in the pupa the genitalia are distinguishable (Fahmy, 1931; Hickin, 1940), and the sex can therefore be determined.

After moulting to the adult the animal does not at once leave the pupal case, but remains in it for some days before eating its way out. This period between moulting and leaving the case is known as the pre-emergence stage. In earlier experiments some difficulty was encountered in determining the end of the pre-emergence stage. If only a thin layer of food is left over the pupal case, then after leaving the case the animal may again return to it; if a deep layer of food is left, it may take the animal a considerable time to make its way to the top, and therefore its appearance at the surface cannot be taken as a criterion of emergence. In later experiments it was found that a satisfactory determination of emergence could be made by leaving the pupal case completely covered with food and marking its position on the outside of the tube. The date on which the animal was seen to be no longer under the mark was then the date of emergence.

In the experiments on duration of development, the animals were examined once ay during the relevant periods, and at about the same time each day. The dura- n of a stage was found simply by subtracting the date of, say, the first discovery an egg from the date of the discovery of its having hatched. This determination he duration might be in error by nearly ± 1 day in a single case, since the egg ght have been laid nearly a day before it was found and have hatched just before vas noted or, alternatively, it might have been laid just before discovery and ched nearly a day before it was noted. In a large number of cases, however, the or of the average from this cause would be much smaller than ± 1 day and would d towards zero, in the absence of systematic error. A systematic error would ur if there were diurnal rhythms of laying, hatching, moulting, etc. with their ks at different times of day; we have no information about this.

III. THE DURATION OF DEVELOPMENTAL STAGES

A. *The effect of temperature*

We are concerned with the effects of temperature and humidity on the duration the developmental stages. These factors would be most satisfactorily dealt with means of a set of experiments covering a complete range of humidities at each the temperatures in the chosen range. This has not been possible, and it was essary to choose a humidity for the temperature experiments. It is shown below t low humidities are deleterious of *Ptinus*; while at relative humidities of over % it is difficult to keep cultures free from moulds. The temperature experiments re therefore done at the highest relative humidity (70 % R.H.) at which freedom m moulds could conveniently be maintained.

The results are shown graphically in Fig. 1, and the figures given in Table 1. ne larval stage has been studied as a whole, and the effect of temperature on the rations of the separate instars has not been dealt with. On wholemeal flour and ast mixture at 22·5° C. and 75 % R.H. there are three instars with average dura- ns of $7{\cdot}2 \pm 0{\cdot}15$, $8{\cdot}6 \pm 0{\cdot}12$, and $20{\cdot}7 \pm 0{\cdot}61$ days respectively. Fahmy (1931) tes, however, that the number of instars is variable.

It will be seen from Fig. 1 that for all four stages—egg, larva, pupa and pre- nergence—the time taken for the completion of the stage falls with rising tem- rature in the usual way to a minimum; it then rises for a short time before the hal temperature is reached. In Figs. 1 and 2 the rate of development expressed $100/T$, where T is the time taken in days for the completion of a stage, has been otted against temperature. It will be seen that the acceleration of development used by an increase in temperature is constant until a certain point is reached, d above this begins to fall off. This point is referred to as the critical temperature. is slightly lower than the temperature at which development takes place in the ortest time. At the latter temperature the accelerating effect of temperature is ready counterbalanced by an opposing effect which first shows itself immediately ove the critical temperature. The critical temperatures obtained from Fig. 1 the four stages are as follows:

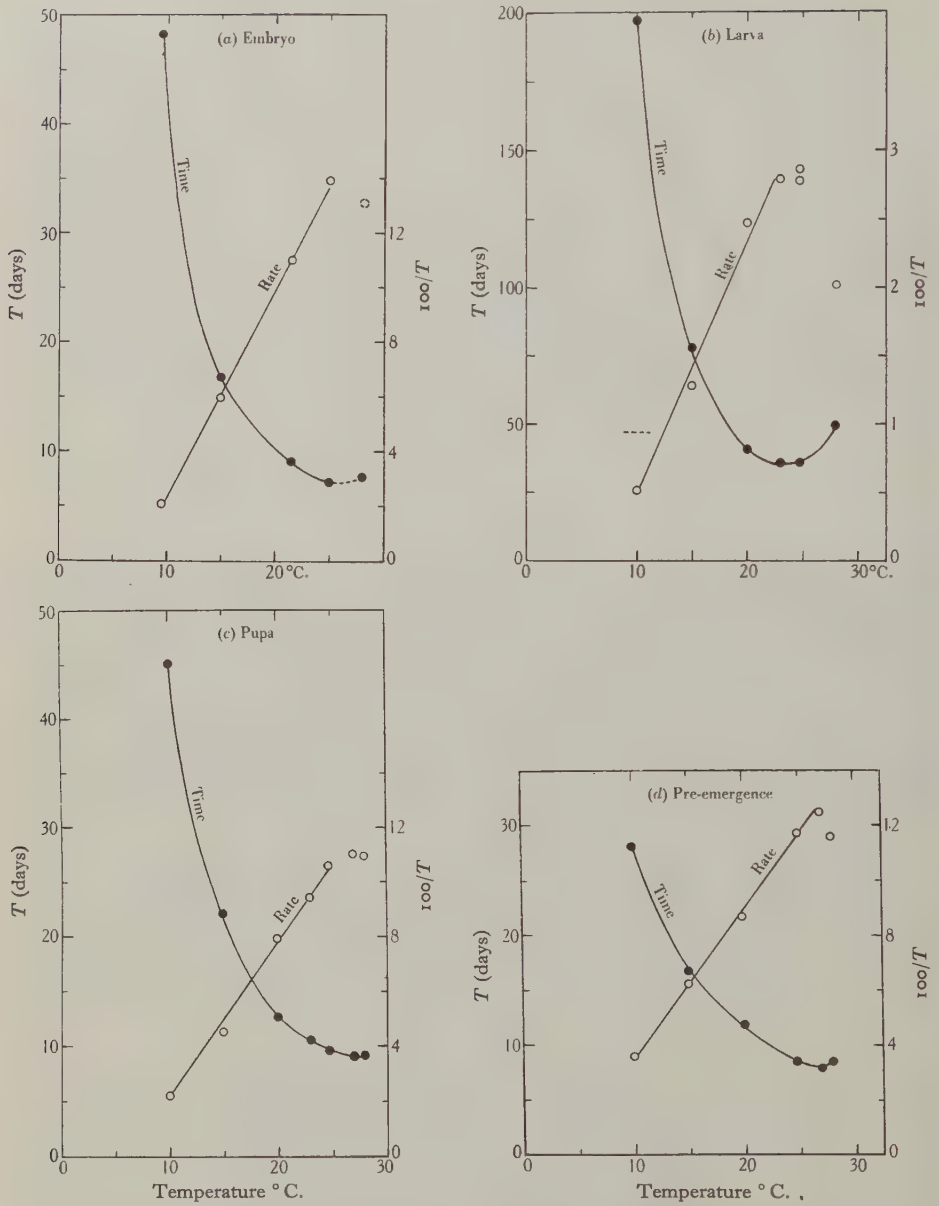

Fig. 1. Development of *Ptinus tectus*, showing the duration (T) and the rate ($100/T$) of development of (*a*) embryonic, (*b*) larval, (*c*) pupal, and (*d*) pre-emergence stages at 70 % R.H. Note that the time scale of (*b*) larval development is one-quarter of that used for the other stages. In (*a*) the broken line joins the final point at 28° C. which is for 100 % and not 70 % R.H. In (*b*) the broken line indicates the upper possible limit for the rate at 10° C., the values for that temperature being estimated as indicated in the text.

Egg	between 25 and 28° C.
Larva	,, 23 ,, 25° C.
Pupa	,, 27 ,, 28° C.
Pre-emergence	,, 27 ,, 28° C.

When considering factors influencing the duration of the egg stage, it should be understood that two processes are involved, embryonic development, and eclosion from the egg. Temperature and humidity need not necessarily have similar effects on the two processes, and it is desirable to differentiate between them. The upper limit of temperature for hatching is lower than that for development, since at 9·5° C. no eggs will hatch although development is not prevented, for the mandibles of the larva can be seen through the shell. At 28° C. and 70 % R.H. the same

Table 1. *The effect of temperature on development at* 70 % R.H.

Temp. (°C.)	*T* (days)	% completed	Number
	Embryonic development		
29·5	∞	0	374
28·0	*7·7 ± 0·25	25	103
25·0	7·2 ± 0·03	93	378
21·5	9·1 ± 0·11	76	87
15·0	16·8 ± 0·05	?	229+
9·5	48·3 ± 0·21	87	143
	Larval development		
30·0	∞	0	9
28·0	49·5 ± 2·20	11	36
24·7	36·0 ± 0·42	95	95
23·0	35·9 ± 0·20	100	61
20·0	40·5 ± 0·52	100	65
15·0	77·9 ± 0·46	95	66
10·0	†197·3	83	61
	Pupal development		
30·0	∞	0	12
28·0	9·1 ± 0·15	89	19
27·0	(i) 9·1 ± 0·02	98	60
	(ii) 9·1 ± 0·11	100	43
24·7	9·5 ± 0·07	99	89
23·0	10·6 ± 0·07	100	61
20·0	(i) 12·8 ± 0·05	100	92
	(ii) 12·5 ± 0·07	100	65
15·0	(i) 22·1 ± 0·07	100	83
	(ii) 22·1 ± 0·14	100	55
10·0	‡45·1 ± 0·85	100	61
	Pre-emergence stage		
30·0	∞	0	?
28·0	8·5 ± 0·36	88	17
27·0	(i) 8·0 ± 0·15	98	58
	(ii) 7·8 ± 0·16	100	43
24·7	8·5 ± 0·12	100	88
20·0	(i) 11·5 ± 0·18	100	92
	(ii) 12·5 ± 0·53	100	65
15·0	(i) 13·8 ± 0·24	100	83
	(ii) 19·7 ± 0·50	100	55
10·0	28·1 ± 1·06	100	61

* R.H. 100 %. † Estimated, see text. ‡ R.H. 52 %.

thing occurs, and the value for the duration of embryonic development at 28° C. used in Fig. 1 *a* corresponds to a humidity of 100 % R.H. If the value for 70 % were used, since there is no hatching, T would be infinity and $100/T = 0$. In either case the critical point will be seen to be below 28° C. Clark (1935) records that at 35 and 36° C. *Rhodnius* eggs fail to hatch, although development takes place, and attributes this failure to hatch to a hardening of the chorion (cf. pp. 8–10 below).

König (1936), on the basis of extrapolation from higher temperatures, states that 15·1° C. is the lower limit of embryonic development. Table 1 and Fig. 1 *a* show that development and hatching occur at least as low as 9·5° C.

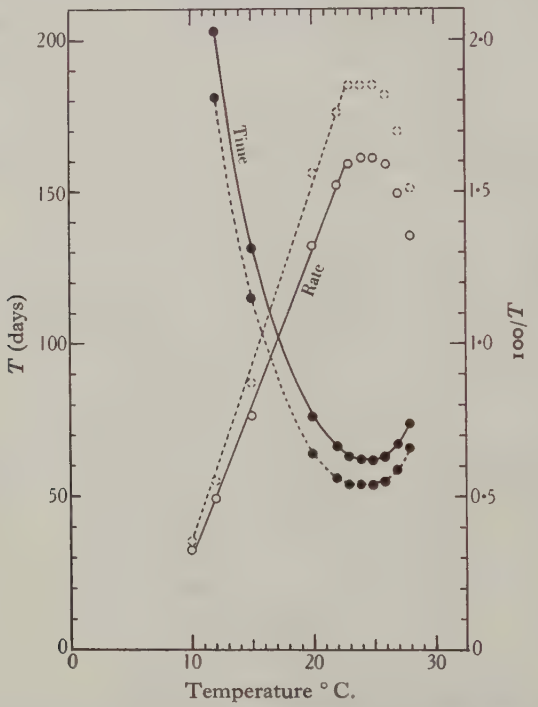

Fig. 2. Duration (T) and rate ($100/T$) of development of *Ptinus tectus* from egg laying to (*a*) the last moult (broken lines) and (*b*) the emergence of the adult from the cocoon (full lines).

Some further explanation of Fig. 1 *b*—larval duration—is necessary. At 24·7° C. two values of $100/T$ are shown. During the course of the experiments the heating failed for 36 hr., and the temperature fell slightly. The value of the larval duration obtained is therefore probably slightly too long. For the lower of the two points the larval duration actually observed was used to calculate $100/T$, and for the higher point 1 day was deducted as an allowance for the time the heating failed. This deduction is almost certainly over generous, but even when this allowance is made it is apparent that the temperature of 24·7° C. is above the critical point for larval development.

The temperature regulation of the experiments at 10° C. also failed slightly over half-way through the experiments, and the animals were allowed to finish their

development at 25° C. From the larval duration so obtained the duration at 10° C. has been calculated and is used in Fig. 1*b*. Obviously the point at 10° C. may be inaccurate, but in any case the larval duration cannot have been shorter than the time taken partly at 100 and partly at 25° C. This gives the upper limit for $100/T$ at 10° C., as shown by the broken line.

Fig. 1 thus shows that the critical temperature is lowest for the larval stage, and highest for the pupal and pre-emergence stages.

By adding together the values for the duration of the various stages found from Fig. 1 the duration of total development at any temperature can be found. These figures are shown graphically in Fig. 2. The duration of development both including and excluding the pre-emergence stage is given. It will be seen that in either case the critical temperature for total development is approximately 23° C. This reflects the fact that the larval stage is the longest of the four and has its critical temperature at about 23° C.

In Table 1 the percentage of animals which complete each stage is given. It will be seen that in all stages the mortality rises at temperatures above the critical point, the effect being most marked in the case of the egg and the larva. Above the critical temperature there is thus both a slowing down of the rate of development, and an increase in the mortality.

B. *The effect of humidity*

The effect of humidity has been studied at various temperatures for the four developmental stages, and the results are given in Table 2.

Humidity has been found to have different effects on embryonic development and on hatching. Low humidity has often been recorded as interfering with the hatching process. Severin & Severin (1910) recorded that in *Diapheromera femorata* at low humidities there was great difficulty in hatching, and many animals were unable to extricate themselves from the egg. Janisch (1930) found that at 29° C. *Prodenia littoralis* eggs would not hatch at 0% R.H. although the eggs contained fully developed larvae, some of which even bit a hole in the shell. Andersen (1930) reported that in *Sitona lineata* the hatching process is slower at low humidities. This effect of low humidity on hatching has not always been taken into account when considering the effect of humidity on the duration of embryonic development. As will be seen from the experiments described below, this effect on hatching may give a spurious appearance of an effect on the duration of embryonic development.

In *Ptinus* at 29·5° C., as mentioned above, there is no hatching at any humidity, although larval mandibles can be seen through the shell. At 28° C. hatching takes place at 100 % R.H., while at 70 % R.H. there is development but no hatching. At 25° C. a decrease in R.H. from 100 to 70 % has only a slight effect, but below 70 % there is a considerable increase in the duration of the egg stage, while at 0 % there is no hatching but development occurs and larval mandibles can be seen. In order to differentiate between the effects of humidity on development and on hatching a series of experiments was therefore done at 26° C. in which batches of eggs were

kept at 100, 30 and 0 % R.H. for 5 days. All the eggs were laid at 26° C. and 70 % R.H. After the 5 days one-third of the eggs at 100 % were transferred to 30 %, one-third to 0 %, and the rest left as controls at 100 % R.H. Half of the batches at 30 and 0 % were transferred to 100 % and the other halves of the two batches left as controls at 30 and 0 %. The periods in days taken for complete hatching in each case were as follows: 100 % controls $7{\cdot}0 \pm 0{\cdot}05$, 100 % transferred to 30 % $8{\cdot}2 \pm 0{\cdot}20$; 30 % controls $10{\cdot}1 \pm 0{\cdot}56$, 30 % transferred to 100 % $7{\cdot}2 \pm 0{\cdot}05$; 0 % transferred to 100 % $7{\cdot}8 \pm 0{\cdot}06$. At 0 % neither the controls nor the eggs transferred

Table 2. *The effect of humidity on development*

Temp. (°C.)	R.H. %	*T* (days)	% completed	Number
		Embryonic development		
25·0	100	$6{\cdot}8 \pm 0{\cdot}04$	?	174+
	70	$7{\cdot}2 \pm 0{\cdot}03$	93	378
	52	$8{\cdot}7 \pm 0{\cdot}06$	66	196
	34	$12{\cdot}3 \pm 0{\cdot}25$	56	119
	0	∞	0	417
		Larval development		
24·7	70	$36{\cdot}0 \pm 0{\cdot}42$	95	95
	52	$47{\cdot}5 \pm 0{\cdot}66$	86	69
	34	∞	0	60
		Pupal development		
27·0	90	(i) $8{\cdot}9 \pm 0{\cdot}08$	85	25
	90	(ii) $9{\cdot}0 \pm 0{\cdot}10$	100	47
	70	(i) $9{\cdot}1 \pm 0{\cdot}02$	98	60
	70	(ii) $9{\cdot}1 \pm 0{\cdot}11$	100	43
	52	$9{\cdot}1 \pm 0{\cdot}08$	97	59
		Pre-emergence stage		
27·0	90	(i) $10{\cdot}1 \pm 0{\cdot}33$	100	21
	90	(ii) $8{\cdot}9 \pm 0{\cdot}20$	100	47
	70	(i) $8{\cdot}0 \pm 0{\cdot}15$	98	58
	70	(ii) $7{\cdot}8 \pm 0{\cdot}16$	100	43
	52	$7{\cdot}7 \pm 0{\cdot}50$	100	59
24·7	70	$8{\cdot}5 \pm 0{\cdot}12$	100	88
	52	$9{\cdot}8 \pm 0{\cdot}17$	97	57

from 100 % hatched, although some of the latter punctured the shell, and in both cases the larval mandibles were visible. These experiments show that only hatching, and possibly the very latest stages of development, are sensitive to humidities down to 30 %. Eggs transferred from 0 to 100 % R.H. took about a day longer to hatch than the controls kept at 100 % throughout. It is not clear whether this delay is a true effect of low humidity on rate of development, or merely represents a lag in the time taken for the new humidity equilibrium to be attained. Evans (1934) noted that in *Lucilia sericata* low humidity hinders hatching. When studying the effect of humidity on embryonic development in the species, he therefore moved eggs from lower humidities to 100 % R.H. as soon as controls at this humidity had hatched, and claimed to have shown a linear relation between saturation deficiency and duration of development. In view of the very short times involved (3 hr. and less), however, it does not seem clear whether his experiments have not merely

demonstrated that the time taken for the egg to come into equilibrium with 100 % R.H. bears a linear relation to saturation deficiency. In *Ptinus* it is not clear whether low humidity acts merely by hardening the shell, thus making it more difficult for the larva to bite its way out, or whether the low humidity also affects the activity of the larva. The viability is certainly reduced at low humidities.

Humidity has a marked effect on the duration of the larval stage (Table 2). The larva is also the stage most adversely affected by low humidity, for at 34% R.H., at least at 24·7° C., the stage is not completed, while all other stages can be completed at this humidity. This is in contrast to *Tribolium*, which can complete its whole cycle of development at 25 % R.H. (Holdaway, 1932).

There may be a slight increase in the duration of the pupal stage with decreasing humidity, but the effects recorded are not significant. There is also little or no effect on the duration of the pre-emergence stage. In one series of experiments (27° C.) a decrease in humidity appears to have the effect of decreasing the length of the stage, while at 25° C. the reverse effect is found. In neither case is the effect large. Table 2 shows that low humidity reduces the viability of egg and larva, but has little effect on that of pupal and pre-emergence stages.

There are no significant sexual differences in the durations of larval, pupal or pre-emergence stages.

IV. THE ADULT

A. *Duration of life*

Hickin (1940) gave figures for the duration of adult life without food at various temperatures and humidities. The duration of adult life under optimal conditions, in the presence of food and with drinking water available, has not been determined. This period, however, must be well over 7 months at room temperature (10–20° C.) for, as mentioned above, animals kept in shallow cultures remained perfectly healthy for this length of time, and during the course of the experiment only one animal died naturally.

The duration of adult life in the presence of food, but without water to drink, has been determined at certain temperatures and humidities. The animals were kept singly or in pairs, one male and one female, in small tubes and examined weekly. Fresh food at the appropriate temperature and humidity was given fortnightly. In all cases the animals had been reared at the experimental temperature and humidity since before pupation. Table 3 gives the time in weeks taken for 50 % of the animals to die, together with the minimum and maximum durations of life. There seemed to be an indication that males may live slightly longer than females, but the data are not sufficient to make this certain.

It will be seen that the duration of adult life is shortened by a rise in temperature or a decrease in relative humidity. Hickin (1940) found a similar effect of humidity and temperature on duration of adult life in the absence of food.

Fahmy (1931) made the statement that 'constant temperature is fatal to *Ptinus tectus*, at least in the adult stage'. In his experiments humidity was uncontrolled

and the temperature varied from 27·5 to 29° C. It seems probable that the death of his animals was due to a combination of high temperature and low humidity rather than to 'constancy' of temperature.

Table 3. *Duration of adult life in the presence of food but without water*

Temp. (°C.)	R.H. %	50 % dead (weeks)	Min. life (weeks)	Max. life (weeks)	Number
27·0	70	(i) 6–7	5	9	52
	70	(ii) 6–7	4	8	38
	50	4–5	3	7	17
20	70	10–11	5	18	91
15	90	25	14	?	58
	70	16	10	36	69
	50	12–13	4	20	30

B. *The effect of free water and of relative humidity on food intake*

The appearance, on dissection, of the animals which had died in the experiments just described suggested that low humidity might have an effect on foot intake, and experiments were therefore carried out to test this. A number of animals were starved at 70 % R.H. and 25° C. for a week. Sample dissections showed that after this period their crops contained no food. The remaining animals were then divided into four lots and placed on food at 90, 70, 52 and 34 % R.H. at 25° C. Ten individuals from each humidity were dissected at intervals of 24, 48 and 96 hr. after being placed on the food. On dissection the number of animals that have eaten can be ascertained, as the crops are then full of food. In a second similar experiment, the animals were given a drink before being put on the food mixture. The averages of two such pairs of experiments are shown in Fig. 3.

It will be seen that at 50 % R.H. the majority of animals ate if they had been given the opportunity of drinking; but when not allowed to drink, less than 40 % of animals ate. At 34 % R.H., even when allowed to drink, the majority of the animals did not eat. It was observed that at 34 % R.H. the crops of most of the animals which had been allowed to drink remained full of water. It therefore appeared possible that at low humidities water might be retained in the crop and prevent the eating of solid food, while at higher humidities the water might be passed on and permit the filling of the crop with food. This was shown not to be the case by the following experiments. Animals were starved as before at 70 % R.H. and then allowed to drink. They were then placed at the two humidities 90 and 34 % without food, and samples dissected at intervals. Water was retained in the crop in all cases for several days, and seemed to be little influenced by the humidity. Had food been present all the animals at 90 % R.H. would have eaten after 24 hr., and therefore we are not dealing with a water retention present only at low humidities which prevents the eating of solid food.

A similar series of experiments was carried out with larvae at the same four humidities. It is difficult to determine whether food is or is not present in the crop

of the larvae since the crop is very thin walled and easily broken, and freshly eaten food does not show up against the white tissues. The food for these experiments was therefore coloured by mixing with it a small quantity of carmine. It was found that food at all four humidities was eaten, but a quantitative difference which could not be accurately assessed existed: the larvae at 90 % ate more than those at 34 % R.H. As mentioned before (p. 299) larvae are unable to complete their development at 34 % R.H.

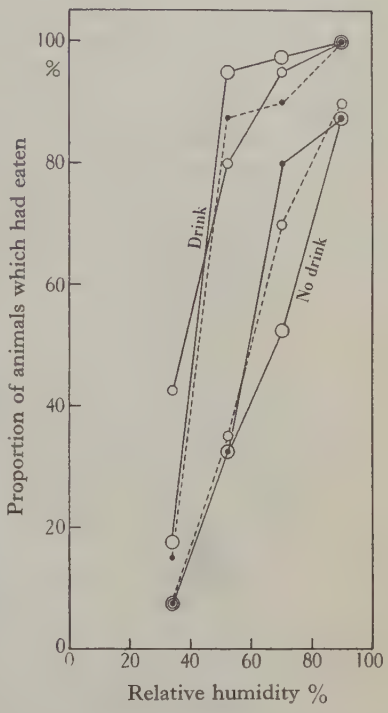

Fig. 3. Proportion of adult *Ptinus tectus* which ate flour at various humidities. Left—animals allowed to drink before test; broken line and solid circles—examined after one day's test; full line and smaller circles—after 2 days; full line and larger circles—after 4 days. Right—animals not allowed to drink; full line and solid circles—after 1 day; broken line and smaller circles—after 2 days; full line and larger circles—after 4 days.

C. *Maturity*

Dissection of freshly emerged adults showed that the males are sexually mature, but the females are not. The testes and sperm sacs of the males contain active sperm, but the ovaries of the females are immature and do not contain ripe eggs. The minimum time for the maturing of the female has not been accurately determined, but at 25° C. and 70 % R.H. it lies between 3 and 12 days after emergence. Since there is no sexual difference in the length of the pre-emergence period, the difference in maturation time of the gonads cannot be accounted for by a longer pre-emergence stage in the male.

The minimum time after emergence taken for impregnation of the female was found to be less than 24 hr. at both 25 and 15° C. This was determined by dissection of the females and the finding of active sperm in the spermatheca. These results do not confirm Fahmy's (1931) statement that a feeding period of at least two weeks was necessary before impregnation could take place.

D. *Oviposition*

A preliminary experiment has shown that water drinking has a great effect on the oviposition rate. Fig. 4 shows the result of two experiments, one at a mean humidity of 60 %, and the other at 45 % R.H. In each experiment the eggs laid by two batches of about twenty animals of undetermined sex were counted daily. No water was offered until oviposition had ceased for some days. Free water was then offered daily to one batch in each experiment. It will be seen that this opportunity for drinking resulted in a rapid rise in the oviposition rates of these two batches, while no further eggs were laid by the controls. These results may explain

the observation of Fahmy (1931) that the average number of eggs laid per female in a lifetime was about 10. Hickin (1940) also states that 'relatively small numbers of eggs are laid by any one female', but records a very considerable increase in oviposition in the presence of free water.

No systematic experiments have been done to measure the oviposition rate, but some idea of the number of eggs laid per adult per day may be gained from experiments made for another purpose. Six batches of between twenty and thirty adults, 127 animals in all, were kept in shallow glass dishes for various periods ranging

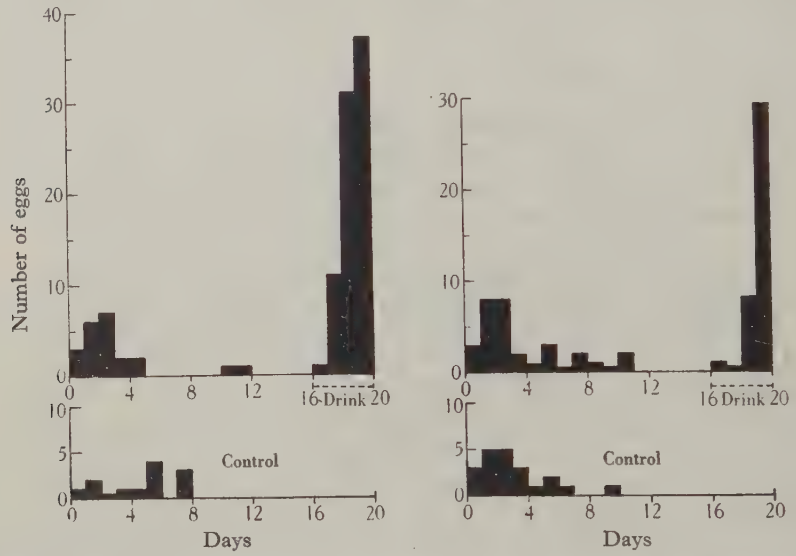

Fig. 4. Effect of water drinking on egg laying of *Ptinus tectus* (two experiments). Below—no water given; above—no water given until the 16th day and then offered daily. Note the outburst of egg laying when drinking was permitted.

from 6 days to 3 weeks at 25° C. The animals were given a drink daily and the humidity was high, about 80 % R.H. The eggs were collected and counted every 24 hr. These experiments gave a mean oviposition rate of 2·8 eggs per adult per day. No allowance was made for the fact that adult *Ptinus* will eat eggs, and thus the oviposition rate recorded may be lower than the true rate. (The egg appears to be the only stage eaten by the adult, but larvae will eat eggs, smaller larvae, and pupae, even in their cocoons.) In one case twenty-six adults showed a mean oviposition rate of 3·5 eggs per adult per day for 15 days. After that the rate fell, but this was probably due to bad culture conditions. The sex ratio was not determined, so that this rate may represent a rate of 3·5 eggs per female, or, according to the number of males present, a higher rate.

The lower limit for oviposition is in the region of 5° C., for a preliminary experiment with about 200 animals which were daily offered a drink of water showed that no eggs were laid at 5° C., while at 7° C. the numbers of eggs laid on successive days were 7, 3, 1, 5, 1, 1.

The upper limit for oviposition is about 30° C. Adults transferred to 30° C. showed a fairly rapid fall in oviposition rate, and accompanying this a fall in the percentage hatch of the eggs laid by adults at 30° C. and moved to 25° C. and 70 % R.H. for hatching. Such an experiment with nineteen adults is shown in Table 4; the adults were kept at 25° C. until a steady oviposition rate was obtained and then removed to 30° C. and kept there. At both temperatures the adults were offered water daily. The first value in Table 4 is at 25° C., the rest at 30° C. The eggs which were laid at 30° C. and developed at 25° C. took the same time to hatch as eggs which had been laid at 25° C. When oviposition has ceased at 30° C., if the culture is returned to 25° C. oviposition recommences, but at a low rate and with low fertility. Dissections of animals exposed for several days to 30° C. showed the gonads to be abnormal.

Table 4. *The effect of high temperature* (30° C.) *on egg-laying*

Day	1	2	3	4	5	6	7	8	9	10	11	12	13
No. of eggs laid	56	31	32	32	39	27	18	32	14	3	5	3	1
% hatch at 25° C.	97	61	62	66	54	41	44	18	7	0	0	0	0

V. DISCUSSION

The present results are acknowledged to be very incomplete. They indicate, however, the great importance of humidity in the economy of the life of *Ptinus* from the standpoint of the hatching of the egg, the growth of the larva, and the oviposition rate, feeding and duration of life of the adult. They also indicate the low upper limit of temperature for development.

It is interesting to compare the values found for the temperature and humidity limits of *Ptinus* with those of other grain pests. Park (1934) gives 32° C. as the temperature at which *Tribolium confusum* completes its life cycle in the shortest time on whole wheat flour at 75 % R.H., while Stanley (1932) gives the same temperature as that at which the oviposition rate is at a maximum. The temperature at 23° C. at which *Ptinus* completes its development in the minimum time is surprisingly low. The minimum temperature at which *Ptinus* can complete its development has not been determined, but it lies below 10° C., while the lower limit for oviposition is between 7 and 5° C. The lower limit for oviposition for *Tribolium confusum* is 16° C. (Dick, 1937), for *Calandra granaria* 12° C. (Müller, 1927) and for *C. oryzae* 10° C. (Tsai & Chang, 1935). *Ptinus tectus* is therefore adapted to moderate temperatures, a fact which accords with the temperate distribution of the animal.

It is desirable that there should be further investigation of the relation of temperature and humidity to oviposition, and of the effect of free water on the duration of adult life of *Ptinus*.

VI. SUMMARY

At 70 % R.H. development of *Ptinus tectus* from egg laying to emergence from the cocoon is minimal at 23–25° C. and takes an average of about 62 days; at 15° C. the time taken is about 130 days. The minimum temperature at which complete development can occur is below 10° C. and the maximum is between 28 and 30° C.; considerable mortality occurs in eggs and larvae at 28° C. and the eggs require a humidity of 100 % R.H. for hatching at this temperature.

At 75 % R.H. and above it is difficult to prevent moulds growing on the food of *Ptinus*. It appears that 70 % R.H. is a satisfactory humidity for all developmental stages, but in air drier than this (at 25° C.) both eggs and larvae show an increased mortality and a prolonged development. The hatching of the egg, rather than embryonic development, seems to be sensitive to low humidity.

Adults given food but not water live longer at 70 % R.H. (27° C.) and at 90 % R.H. (15° C.) than at lower humidities. At 25° C., below 70 % R.H. feeding is much reduced if water to drink is not given; at 34 % there is little feeding even when free water is available. Larval feeding is also reduced at low humidities. Oviposition soon ceases unless drinking water is available, at any rate at low humidities.

Ptinus tectus is thus adapted to a temperate climate with a high humidity.

Our thanks are due to Prof. J.W. Munro of the Imperial College of Science in whose department some of the experiments were completed; to Dr O.W. Richards for suggesting the problem and for very useful advice; to Dr D. L. Gunn for directing the work in its earlier stages, for helpful criticism throughout, and for assistance in providing apparatus; and also to Dr Gunn and Miss B. M. Walshe for tending experiments when neither author could be present to observe; to Dr N.E. Hickin for specimens of *Ptinus* and for information about culture methods, and to Dr J.G. Hay of Messrs Ranks Ltd. for advice about suitable flours and other points.

During the course of this work one of the authors (R.F.E.) was Owen Research Fellow in Zoology at the University of Birmingham.

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CHROMATIC BEHAVIOUR OF *SCYLLIUM CANICULA*

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(With Five Text-figures)

I. INTRODUCTION

CHROMATICALLY active forms are found in all classes of cold-blooded vertebrates. Changes in skin pigmentation result from 'expansion' and 'contraction' of dermal and epidermal chromatophores. Dermal melanophores are usually the most important in transition from the pale to the dark condition and vice versa. Most species exhibit two kinds of melanophore response to light: (*a*) a primary response of melanophore expansion in light; (*b*) a secondary response of melanophore expansion to overhead illumination in non-reflecting surroundings (black background) and of melanophore contraction to overhead illumination in surroundings which reflect light (white background). The eye is the receptor for the secondary response. The primary response is non-visual. The range of the primary is usually, but not always, slight in comparison with that of the secondary response. With regard to the control of the latter, it is customary to distinguish between humoral and direct nervous co-ordination of the melanophores. Recent work has shown that it is more precise to distinguish between species of two types:

(*a*) those which exhibit humoral control with no direct peripheral nervous intervention;

(*b*) others in which direct nervous control is superimposed to a greater or lesser extent on the more archaic humoral mechanism.

Hogben and Winton (Hogben, 1924) investigated the effect of hypophysectomy, injection of extracts, drugs, nerve section and nerve stimulation on the chromatic behaviour of Amphibia. They concluded that the hypothesis of 'pituitary secretion fluctuating in correspondence with the action of natural stimuli. . . is in the existing state of knowledge adequate, at least in adult amphibia, to interpret all the salient facts'. Lundstrom & Bard (1932), Hogben (1936), Parker (1937), Wykes (1936), and Waring (1936*a*, 1938) have since shown that elasmobranch melanophores also are under pituitary control. When Hogben's monograph was published (1924) the available data on melanophore expansion and contraction could be explained in terms of increased or reduced secretion of the melanophore expanding hormone.

In 1931, Hogben & Slome published results of an extensive investigation of the chromatic behaviour of *Xenopus laevis*. They postulated that the chromatic behaviour of this animal depends upon fluctuation of two antagonistic hormones, *B* ('expanding') and *W* ('contracting'). *B* is the very stable melanophore-expanding hormone from the intermediate lobe of the pituitary. *W* has not been identified with properties of gland extracts. Evidence for its existence comes mainly from three classes of experiments (Hogben & Slome, 1931, 1936):

(*a*) time relations of the responses of intact animals;

(*b*) responses evoked by the separate removal of the various lobes of the pituitary;

(*c*) differential tolerance of different classes of normal and operated pale animals to equal injections of *B*-containing extracts.

Similar but less complete evidence has been derived from studies on elasmobranchs (Hogben, 1936; Waring, 1936*a*, 1938) and *Anguilla* (Neill, 1940; Waring, 1940; Waring & Landgrebe, 1941). To explore more fully the issues raised in these investigations, the writers planned an investigation of the chromatic effector response of *Scyllium* with the object of (*a*) comparing its behaviour with that of *Xenopus* and *Anguilla*; (*b*) carrying out experiments of a new type to throw light on the ultimate fate of the *B* hormone in the animal body. Wartime restrictions led to withdrawal of boat permits when many of the experiments were incomplete; and there seems little likelihood of obtaining further supplies of fish in the near future. What follows is therefore an interim report on a theme which merits fuller treatment.

Waring (1938, 1942) and Abramowitz (1939) have already reviewed previous work on elasmobranchs. It includes hypophysectomy, injection of pituitary extracts, action of drugs, nerve section and nerve stimulation. About the normal responses of elasmobranchs we still know too little. There are no available data concerning time relations of intact fish during transition from complete darkness to white and black backgrounds with overhead illumination and vice versa.

II. RESPONSES OF INTACT *SCYLLIUM* TO CHANGE OF BACKGROUND AND COMPLETE DARKNESS

Unless kept in large tanks with abundant running water, elasmobranchs will not survive for long periods in captivity. The need to feed and clean in complete darkness is therefore a serious practical problem. The following procedure was adopted. Fish were caught on lines and brought into the laboratory in churns. They were stored in a large indoor concrete tank with completely light-tight cover. The fish were fed once a week with fresh herring and the tank was flushed out the following day. Both these operations were performed at dusk. To avoid even short exposure to diffuse light the fish were starved for 14 days before the beginning of the experiment. No animal was used until it had been under these conditions for at least a month. This precaution was taken to ensure complete equilibration. The tanks for providing white and black 'backgrounds' were supplied with abundant running water. An incandescent gas lamp was placed at a fixed distance above each

tank. Responses of the dermal melanophores are recorded in accordance with the Hogben scale.

Table 1 shows the mean equilibration values of *Scyllium*.

Table 1

	No. of fish	Melanophore index (μ)
On white background in light	15	1·4
On black background in light	15	4·9
In total darkness	15	2·9

The responses to change of background, etc. are summarized in Figs. 1, 2, 3 and Table 2.

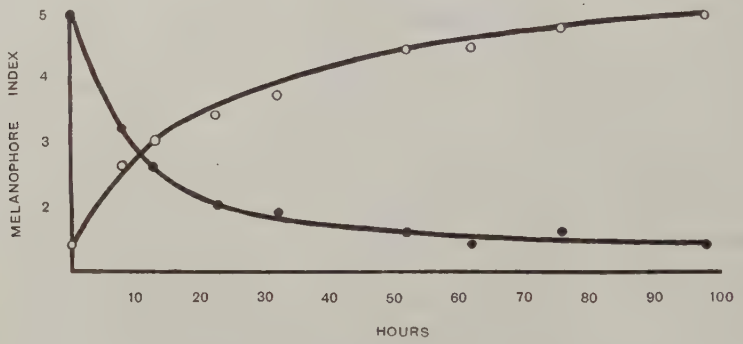

Fig. 1. *Scyllium canicula*. Background reversal in light. 16° C. Each point is average m.i. from six fish.

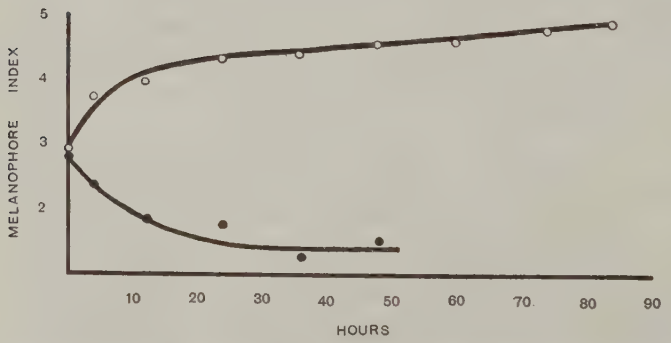

Fig. 2. *Scyllium canicula*. Transition from complete darkness to white and black tanks with overhead illumination. 15–16° C. Each point is average m.i. from six fish.

The time graphs are strikingly similar to those of *Xenopus* and *Anguilla*. Colour change of all three is slow. They all equilibrate at an intermediate condition in complete darkness. In dogfish the prolonged ${}_wT_d$* is similar to that of the other

* Notation adopted by Waring (1938) and Neill (1940) at Hogben's suggestion: ${}_bT_w$ = time taken to change from equilibrium on *black* background in light to equilibrium on *white* background in light, ${}_dT_b$ = time taken to change from equilibrium in complete darkness to equilibrium on black background in light, etc.

two species. We can summarize the chief points of difference between *Xenopus*, *Anguilla* and *Scyllium* as follows:

Xenopus	*Anguilla*	*Scyllium*
${}_wT_b < {}_bT_w$	${}_wT_b = {}_bT_w$	${}_wT_b > {}_bT_w$
${}_dT_b < {}_dT_w$	${}_dT_b < {}_dT_w$	${}_dT_b > {}_dT_w$
${}_dT_b < {}_bT_d$	${}_dT_b > {}_bT_d$	${}_dT_b > {}_bT_d$

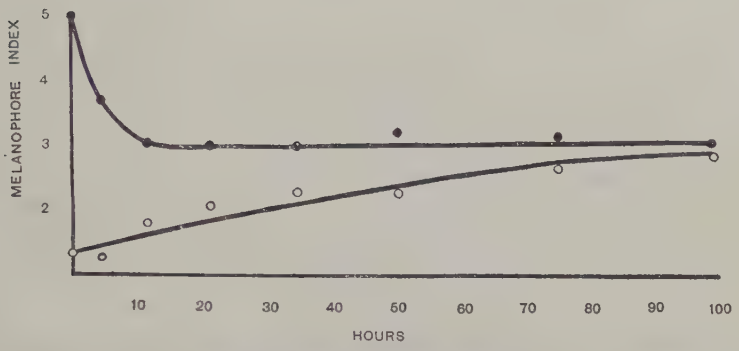

Fig. 3. *Scyllium canicula*. Transition from black and white tanks with overhead illumination to complete darkness. 15–16½° C. Each point is the average m.i. from six fish.

Table 2

	μ range	Time (hr.)
${}_bT_w$	5·0 1·5	60
${}_wT_b$	1·5 5·0	100
${}_dT_b$	3·0 5·0	85
${}_bT_d$	5·0 3·0	12
${}_dT_w$	3·0 1·5	35
${}_wT_d$	1·5 3·0	100

So far *Scyllium* is the only species for which ${}_wT_b$ is greater than ${}_bT_w$ and for which ${}_dT_b$ is greater than ${}_dT_w$. There is independent evidence that *B* is eliminated or destroyed very rapidly in this fish. An injection of *B* sufficient to evoke complete darkening (Waring, 1938) is removed from circulation much more rapidly than in *Xenopus* and *Anguilla*.

III. SIGNIFICANCE OF TIME

Hogben (1924), Hogben & Slome (1931, 1936) and Neill (1940) emphasize the importance of studying time relations before drawing far-reaching conclusions as to the nature of the co-ordinating mechanism concerned. In a co-ordinated response of the type under consideration, a complete cycle of behaviour involves (*a*) time taken for the stimulus to act on the eye, (*b*) time taken for the impulse to reach the pituitary, (*c*) time taken for the pituitary secretion to reach (or fall below) an excitant level in the circulation and (*d*) time taken for the effector to execute its response. The time for (*a*) and (*b*) may be taken as under a minute and any excess within the period of the complete cycle must be due to (*c*) or (*d*). (*d*) can be measured independently and, if significant, we can make due allowance for it.

The melanophore speed of Scyllium canicula

Waring & Landgrebe (1941) investigated the rates of melanophore contraction and expansion of *Anguilla* and *Xenopus*. They perfused whole preparations with (*a*) physiological saline and (*b*) saline containing either posterior lobe pituitary extract or adrenalin. The state of the melanophores was frequently under observation.

In preliminary experiments with *Scyllium* we used a similar technique. Owing to shortage of animals we were forced to adopt an alternative method. The fish remained for long periods in black tanks with overhead illumination. In these circumstances all the melanophores were fully expanded ($\mu = 5$). Rectangular pieces were cut from the edge of the pectoral fin. The cut edge of the skin was separated from the underlying muscle and skeletal elements and the whole dorsal skin with melanophores stripped free. Small pieces were observed microscopically in excavated glass blocks with abundant saline. The saline was changed frequently. When the melanophores were fully contracted, pituitary extract was added to the saline. The graph (Fig. 4) represents the results of one experiment. Each point is the average reading of the melanophores on four pieces of skin taken from the same animal. The lowest reading on the graph is $\mu = 2$. In about half of the experiments a lower figure (1·5) was obtained, but in all cases the lowest reading was reached within approximately 30 min.

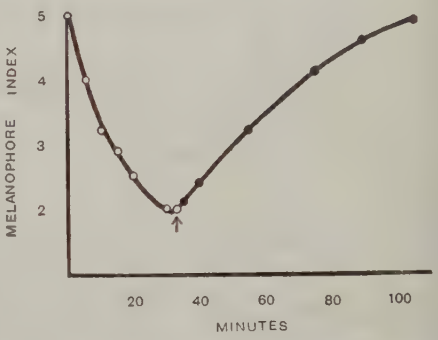

Fig. 4. *Scyllium canicula*. Pigmentary effector speed. 16° C. —○— skin immersed in elasmobranch Ringer. —●— skin immersed in elasmobranch Ringer plus pituitary extract. ↑ = addition of pituitary extract to saline solution. Each point is the average m.i. from four pieces of skin under identical conditions.

The melanophore speed of *Scyllium* as of *Xenopus* (Waring & Landgrebe, 1941) and *Rana* (Waring & McLeod, unpublished) is slow. A comparison of Fig. 4 with Figs. 1, 2 and 3 shows that *the slow effector speed is not the limiting factor in any of the normal responses of the intact animal.* Hence we are justified in interpreting the various time relations ($_bT_d$, $_bT_w$, etc.) as indices of *delay in the process of co-ordination.*

IV. NATURE OF THE CO-ORDINATING MECHANISM

Prior to the work of Hogben & Slome on *Xenopus* it was generally believed that only one pituitary hormone is involved in chromatic behaviour. This view is still that of some authors, and is usually referred to as the *one-hormone hypothesis*. According to the one-hormone hypothesis, production (or release) of the melanophore expanding hormone (*B*) by the pituitary is inhibited on a white background and augmented on a black background in light. This implies that the hormone concentration is highest on a black background ($\mu = 5$), least on a white background

($\mu = 1$) and intermediate in darkness ($\mu = 3$). Hogben believes that this hypothesis is incorrect for the following reasons (inter alia). If it is correct, ${}_wT_b$ is the time taken for *maximum* increase in concentration of hormone; and both ${}_wT_d$ and ${}_dT_b$ involve *smaller* increases in concentration. So neither ${}_wT_d$ nor ${}_dT_b$ can be greater than ${}_wT_b$. Similarly, ${}_bT_w$ is the time taken for *maximum* decrease in concentration of hormone. So neither ${}_bT_d$ nor ${}_dT_w$ can be greater than ${}_bT_w$. These deductions do not agree with observation. In *Xenopus* and *Anguilla* ${}_wT_d$ is greater than ${}_wT_b$. In *Anguilla* ${}_dT_w$ is greater than ${}_bT_w$. In *Scyllium* ${}_wT_d$ is the only time interval which conflicts with the requirements of the one-hormone hypothesis.

The alternative hypothesis suggested by Hogben postulates a second hormone (*W*) which is antagonistic to *B*. The existence of a contracting hormone (*W*) permits of a rational interpretation of the observed time relations (Hogben & Slome, 1931, 1936; Neill, 1940 and Waring, 1942).

For many animals it is established that the background response is due to the stimulation of localized photoreceptors (v. Frisch, 1911; Sumner, 1933, 1940; Hogben & Slome, 1936; Butcher, 1938; Hogben & Landgrebe, 1940). Practical difficulties have prevented similar work on elasmobranchs. In the absence of evidence to the contrary we may assume that

(*a*) photic stimulation of ventral retinal elements evokes release of *B* hormone into the circulation;

(*b*) stimulation of dorsal retinal elements either inhibits the release of *B* hormone (one hormone hypothesis) or evokes secretion of a contracting hormone *W* (bihumoral hypothesis).

Table 3 shows time relations set forth in Figs. 1, 2 and 3 tabulated in relation to the two hypotheses.

We can examine the claims of the two hypotheses from a different angle. If stimulation of the dorsal retinal elements inhibits the secretion of *B* into the circulation, such inhibition may take place in one of two ways: (*a*) inactivation of anabolic activity in the secretory elements; (*b*) prevention of release of the finished

Table 3

	One-hormone hypothesis	Two-hormone hypothesis
1. ${}_wT_b$	*Maximum* increase of *B* due to *release* from reflex inhibition by *dorsal* retinal elements	*Simple* decrease of *W* (while *B* remains constant. *B*: *W* ratio increasing)
2. ${}_bT_w$	*Maximum* decrease of *B* due to reflex inhibition by *dorsal* retinal elements	*Simple* increase of *W* while *B* remains constant. *B*: *W* ratio diminishing
3. ${}_bT_d$	Decrease of *B* due to non-stimulation of *ventral* field	Decrease of *B*
4. ${}_dT_b$	Increase of *B* due to stimulation of *ventral* retinal field *alone*	Increase of *B*
5. ${}_dT_w$	*Sub-maximum* decrease of *B* due to *prepotent* reflex inhibition of *B* through stimulation by dorsal field	*Concomitant* increase of *B*, and of *W*
6. ${}_wT_d$	*Sub-maximum* increase of *B* due to release from prepotent reflex inhibition by dorsal field	*Concomitant* decrease of *B*, and of *W*

product. If (*a*) is true, the one-hormone hypothesis implies that the gland of an animal equilibrated on a white background contains little *B*. So transition to darkness involves the *build up of B both in the gland and in the circulation* without retinal stimulation. We should expect this to be relatively slow. If (*b*) is correct, the one-hormone hypothesis implies that the gland of a fish equilibrated at $\mu = 1$ contains excess of *B*. Transition to darkness therefore involves *release into the circulation of B already stored in the gland.* We should naturally expect this to be rapid. So we can furnish a plausible interpretation of ${}_wT_d$ in terms of one hormone if the glands of fish equilibrated on a white background contain little *B*; and we can put this conclusion to a direct test.

Accordingly, fish were kept in complete darkness, and in white or black tanks with overhead illumination (13° C.). After 10 days the melanophores were read, the fish decapitated and the pituitaries immersed in acetone. Glands from animals kept in darkness were dissected in very dim light. The *B* content of each gland was determined and is expressed (Table 4) in the melanophore unit proposed by Landgrebe & Waring (1941).

Table 4

No. of fish	Tank	Dry weight of neuro-intermediate lobe in mg.*	Total L.W. melanophore units in neuro-intermediate lobe	L.W. units per mg.
1	Complete darkness	2·5	200	80
2	do.	2·2	180	82
3	do.	—	200	—
4	White tank with overhead illumination	2·9	50	16
5	do.	3·2	300	94
6	do.	2·0	240	120
7	Black tank with overhead illumination	2·6	150	57
8	do.	2·0	120	60
9	do.	—	209	—

* On a balance accurate to 0·05 mg.

The results are sufficient to indicate that there is a considerable store of *B* in the glands of fish kept on a white background. Previous work (Waring, 1936*b*) has shown that there is sufficient *B* in the neuro-intermediate of *Scyllium* from illuminated tanks fully to darken four pale fish. It is therefore extremely difficult to believe that the transition from an illuminated white background to darkness involves only the release of inhibition so that the stored *B* can enter the blood stream. If, then, we are prepared to interpret the chromatic behaviour of *Scyllium* in terms of one hormone, we must also conceive of more intricate optic-hypophysial connexions than what we can infer from available experimental evidence.

Many workers subject pituitary extracts and blood samples to caustic soda treatment before assaying their *B* content (Kleinholtz & Rahn, 1940; Levinson, 1940). Levinson estimated *B* activity from the *length of time* that pale animals remained

dark after injection. The degree of potentiation after caustic soda treatment is not necessarily correlated with the *B* content of the untreated material. So assays made after such treatment may be misleading. Our own assays were made on simple aqueous extracts. This is justified because elasmobranch glands contain little or no pressor substance. Caustic soda destroys pressor and oxytocic activity and modifies the melanophore excitant properties. Landgrebe & Waring (1941) have reviewed the literature and made new observations on this modification. Their chief conclusions were

(1) Caustic treatment of whole posterior lobe extracts increases (*a*) expanding power of the extract and (*b*) duration of the response evoked by sub-maximal doses.

(2) Destruction of the pressor autacoid is *not* the prime cause of these effects.

(3) Caustic soda does *not* modify the *B* molecule itself.

(4) 1 (*b*) certainly and probably 1 (*a*) also are due to the modification of some constituent of posterior lobe extract other than *B* or pressor.

In 1933 Koller and Rodewald observed that pituitaries from frogs kept in total darkness have a lower *B* content than those from frogs in an illuminated environment. Jores (1934) confirmed this result but claimed that after caustic soda treatment of the two extracts the difference disappeared. He suggested that the gland of a frog kept in darkness contains a store of *precursor B* and that the latter is activated by caustic soda. Assays of dogfish glands (Table 4) do not suggest a substantial reduction of *B* content in darkness. We ourselves have also tested the effect of caustic soda on glands from fish kept under different conditions. After the assays already recorded the remainder of the pulverized neuro-intermediate lobes from numbers 1, 2 and 3 were thoroughly mixed and a weighed quantity extracted with boiling water. After filtration the extract was divided into two equal parts. Both were made up to *N*/10 with normal caustic soda. One was immediately neutralized with *N* HCl and both were boiled in a water bath for 10 min. After cooling, the extract in the second tube was neutralized. Material from glands 4, 5, 6 and 7, 8 and 9 were similarly treated. The six extracts were assayed by injection into *Xenopus*. Landgrebe & Waring (1941) found that caustic soda treatment of extracts of dogfish glands results in a fourfold increase of potency. In sub-maximal responses such treatment prolongs the duration of response. In the present series we found no difference between the three groups either with respect to degree of potentiation or to duration of response.

V. EFFECT OF THYROIDECTOMY

The effect of thyroidectomy on chromatic function was investigated for two reasons:

(*a*) *B* is probably a complex polypeptide usually bound to a protein. Thyroxine acts on the cells themselves, primarily in facilitating changes in protein metabolism (Mansfield, 1935), and therefore possibly destruction of *B* in the tissues (cf. Landgrebe & Waring, 1941).

(*b*) Hogben & Slome (1936) found that after anterior lobe removal *Xenopus* is less tolerant to *B* substance. They postulated that the pars glandularis secretes a

substance *W* antagonistic to *B*. This differential tolerance has been confirmed (*Scyllium*, Waring, 1938; *Anguilla*, Waring, 1940). Abramowitz (1939) suggested that these results might be due to a 'differential metabolism of the injected *B* hormone'. The metabolic rate of mammals and frogs (Winton & Hogben, 1923) is in fact lower after hypophysectomy, and the fall may be largely attributed to diminished thyroid activity.

Experiments designed to explore the relation of the thyroid to chromatic co-ordination were of two kinds:

(i) *Effect of lighting conditions and hypophysectomy on the thyroid*

If photic stimuli affect thyroid activity they presumably do so by pituitary mediation as with the gonads (Marshall, 1936; Rowan, 1938). There is abundant evidence that the A.L.P. controls the thyroid of mammals, reptiles and amphibia. We have not encountered reports of similar work on elasmobranchs. So we examined the thyroids of three fish which had been hypophysectomized 1 month previously.

To test whether lighting conditions affect the thyroid the following experiment was set up. Three groups each of three intact fish were used. One group was kept in absolute darkness, a second group in illuminated white tanks, the third group in illuminated black tanks. After 8 days the fish were killed. The thyroid gland was dissected out, weighed and fixed in Bouin. Relevant data with reference to the size of the gland are in Table 5.

Table 5

	Melano-phore index	Sex	Weight of fish gm.	Length cm.	Thyroid Wet wt. (mg.) per kilogram body wt.
A	Intact in	3·5	Male	590	61	41
B	complete	3·0	Male	540	58	42
C	darkness	3·0	Female	515	57	97
D	Intact in	1·5	Male	370	51	40
E	illuminated	1·5	Male	450	52	44
F	white tank	1·5	Female	1150	68	91
G	Intact in	5·0	Male	670	66	23
H	illuminated	5·0	Male	480	55	65
I	black tank	5·0	Female	640	59	246
J	Completely	1·0	Female	590	58	161
K	hypophys-	1·0	Female	928	66	98
L	ectomized	1·0	Female	590	62	52

(A to I inclusive are the same fish as 1–9 inclusive of Table 4)

The thyroids were cut into complete serial sections. There was no consistent histological difference between glands of the different groups. Morphological signs of inactivity can be observed in mammals 1–2 weeks after hypophysectomy (Rowlands, 1935). Times for the involution of mature cold-blooded animals are not numerous. Adams & Martindale (1936) found that activity was completely suspended 5 weeks after hypophysectomy of *Triturus*. The epithelium of the glands from hypophysectomized *Scyllium* showed definite signs of activity. So either the

A.L.P. has no control over its activity or elasmobranch glands take longer to undergo involution than other species after removal of the pituitary stimulant.

(ii) *Effect of thyroidectomy*

The elasmobranch thyroid is accessibly situated immediately ventral to the union of the first afferent branchial arteries with the ventral aorta. The main body of the thyroid is a discrete mass attached to the surrounding tissues by a dozen or more distinct strands of connective tissue containing blood vessels. At the anterior end the thyroid is drawn out into a tongue which extends forward towards the union of the two Meckels cartilages. In all our experiments the whole gland was removed.

Thyroidectomized fish are capable of chromatic response. Table 6 shows the equilibration values under different conditions. Fig. 5 is the time graph of transition from white to black background and vice versa.

Table 6

	No. of fish	Average melanophore index
Complete darkness	6	2·1
White tank } Overhead	12	1·1
Black tank } illumination	12	4·3

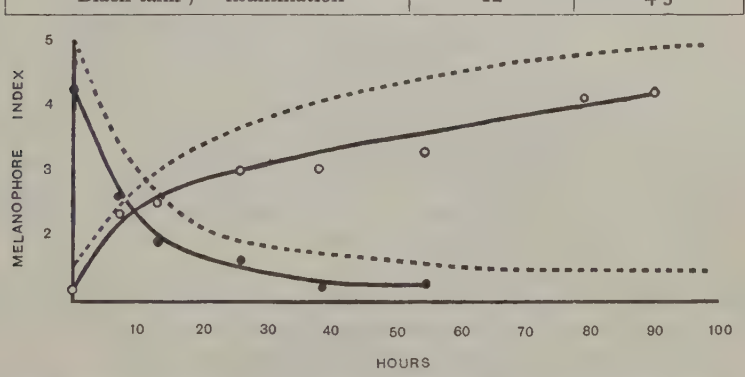

Fig. 5. *Scyllium canicula.* Background reversal of intact and thyroidectomized fish in light. Each point is the average m.i. from six fish. ——— thyroidectomized fish (14–15° C.). ----- intact fish (16° C.).

The data show:

(*a*) equilibration values of operated fish are lower than normal;

(*b*) the speed of response in either direction is approximately the same in operated and intact animals.

Observations on transition from complete darkness to black background with overhead illumination and vice versa were made on only three fish. They indicate no striking difference between normal and operated animals.

These findings are open to several interpretations. They lend no support to the view that thyreo-globulin facilitates the destruction of *B*. If it does, its action is

overruled by other agencies in this class of experiment. In any case, the data recorded do not support the objection of Abramowitz to the two-hormone interpretation of differential tolerance, after total or partial hypophysectomy.

SUMMARY

1. Dermal melanophores of intact *Scyllium* equilibrate at $\mu = 4{\cdot}9$ and $\mu = 1{\cdot}4$ respectively on black and white background with overhead illumination. They equilibrate at $\mu = 2{\cdot}9$ in complete darkness.

2. Normal responses are of the 'slow' type.

3. Expanded melanophores in skin strips immersed in saline contract in 30 min. In pituitary extract they expand in 60 min. So we may interpret the slow responses of intact fish as indices of the slow rise and fall of co-ordinating hormones in circulation.

4. The time relations of normal responses are not consistent with a one-hormone hypothesis.

5. Thyroids removed from animals hypophysectomized a month previously afford no evidence for pituitary control of thyroid activity.

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